REMARKS

Claims 1, 4-26 and 35-38 are pending. Applicants have amended Claims 1, 11, and 19. The amendments add no new matter and are fully supported by the specification and claims as originally filed. Support for the amendments to the claims can be found, for example, in paragraphs [0036], [0041], [0042], [0043], [0050], , [0051], and elsewhere throughout the specification as originally filed.

Claims 1, 4, 7, and 8 were examined in the final Office Action mailed November 12, 2009, and stand rejected. Applicants address the specific rejections set forth in the final Office Action below. For the following reasons, Applicants respectfully traverse.

Rejection Under 35 U.S.C. § 103(a) – Ho et al. and Gold et al.

The Examiner has maintained the rejection of Claims 1 and 4 as allegedly being unpatentably obvious under 35 U.S.C. § 103(a) in over Ho et al. (2002) *Angew. Chem. Int.* 41:1548-1551 in view of Gold (1995) *JBC* 270:13581-13584, for the reasons set forth in the Office Actions mailed June 18, 2009 and October 7, 2008. In the previous Office Actions, the Examiner argued that Ho teaches an optical sensor comprising a ssDNA complementary to a target (*i.e.*, a complementary ssDNA molecule) and the same, water-soluble, cationic polythiophene derivative of the formula shown in Claim 1. The Examiner conceded that Ho does not specifically teach that the ssDNA of the optical sensor is an aptamer, *i.e.* such that the optical sensor detects and aptamer/target complex. The Examiner stated that Gold teaches that aptamers are single-stranded DNA molecules that interact with target molecules and that aptamers can be modified with visualization-enhancing adducts to detect target proteins. In the instant Office Action, the Examiner asserts that complementary binding interactions, *e.g.*, those undertaken by aptamers and their ligands, require electrostatic interactions, hydrophobic interactions and van der Waals interactions. The Examiner argues that one skilled in the art would expect a binding of an aptamer to its ligand would have an optically detectable effect on the electrostatic interaction of the aptamer with a polythiophene. The Examiner concluded that it would have been obvious for one skilled in the art to substitute aptamer nucleic acid sequences as taught by Gold with the polythiophene derivative taught in Ho for the purpose of detecting target molecules recognized by aptamers.

Applicants respectfully disagree. To establish a *prima facie* case of obviousness, the Examiner must establish at least three elements. First, the prior art reference (or references when combined) must teach or suggest all of the claim limitations: "All words in a claim must be considered in judging the patentability of that claim against the prior art." *In re Wilson*, 424 F.2d 1382, 165 U.S.P.Q. 494, 496 (CCPA 1970); *see also M.P.E.P. § 2143.03.* Second, there must be a reasonable expectation of success. *In re Merck & Co., Inc.*, 800 F.2d 1091 (Fed. Cir. 1986); *see also M.P.E.P. § 2143.02.* And finally, the Examiner must articulate some reason to modify or combine the cited references that renders the claim obvious.

Applicants respectfully submit that the cited references cannot support a *prima facie* case of obviousness as required by 35 U.S.C. § 103(a), as the combined teachings of Ho and Gold would not provide the skilled artisan with a reasonable expectation of success, and the skilled artisan would have no reason to modify the teachings of Ho to arrive at Applicants' presently claimed invention.

Claim 1 is drawn to an optical sensor comprising an aptamer and a polythiophene for detecting a target "selected from the group consisting of potassium ions, small organic molecules, amino acids, proteins, whole cells and nucleotides." As discussed during the telephonic interview of January 19, 2010, Applicants respectfully submit that the "nucleotide" aptamer target recited in Claim 1 is not an oligonucleotide, or nucleic acid molecule, as suggested in the final Office Action. (See, final Office Action, p. 4). As such, the optical sensor of Claim 1 is not the same as the optical sensor described in Ho, as suggested by the Office Action.

The Ho reference relates to the optical detection of oligonucleotides that form dsDNA hybrids with complementary oligonucleotides. In the presence of a polythiophene, the dsDNA and the polythiophene form a triplex structure (dsDNA/polythiophene). (See, Ho, Figure 2). Applicants' presently claimed optical sensor differs from the optical sensor in the Ho reference as the presently claimed optical sensor is used to detect the specific quadruplex complexes formed by aptamers and their cognate targets, wherein the target is selected from the group consisting of potassium ions, small organic molecules, amino acids, proteins, whole cells and nucleotides.

The Examiner's 103(a) rejection is based upon the premise that the ability to detect interactions between two complementary oligonucleotides with a polythiophene, as discussed in

Ho, can be extrapolated to any and all aptamer/target interactions. The Examiner contends that the skilled artisan would have a reasonable expectation of success, based upon the assertion that polythiophenes are molecules that are "sensitive to minor perturbations." As explained in further detail below, the skilled artisan would have had no expectation polythiophenes could be used in optical sensors to detect aptamer/target interactions, prior to Applicants' disclosure in the instant specification given the state of the art.

As evidence of the state of the art regarding optical detection as of the effective filing date, Applicants submit herewith as **Exhibit A,** a Declaration from Mario LeClerc, Ph.D., an expert in the field of material chemistry, and a Canada Research Chair on Electroactive and Photoactive Polymers and a named inventor on the above-captioned application. Dr. LeClerc describes the detection of single stranded nucleic acid molecules (*e.g.*, oligonucleotides) in a solution using a polythiophene and a complementary ssDNA, as described in Ho. Dr. LeClerc affirms that it was known that polythiophenes are capable of forming a "triplex" complex with double stranded DNA. (LeClerc Decl., ¶5) According to Dr. LeClerc, during the formation of the complex the polythiophene alters its structure, resulting in an optically detectable signal. (See, *Id.*). The "triplex" complex and its optical signal are also described in Ho. (See, Ho, Figs. 1-2).

As of the effective filing date, the structure of aptamer/target complexes was understood, and it was known that they form G-quartet quadruplexes. The G-quadruplex structure of aptamer/target complexes is described, for example, in Osborne et al. (1997) *Chem. Rev.* 97(2):349-370, submitted herewith as **Exhibit B**, Nutiu, et al. (2003) *J. Am. Chem. Soc.* 125(16):4771-4778, submitted herewith as **Exhibit C**, Vairamani, et al. (2003) *J. Am. Chem. Soc.* 125(1):42-43, submitted herewith as **Exhibit D**, Michaud, et al. (2003) *J. Am. Chem. Soc.* 125(28):8672-8679, submitted herewith as **Exhibit E**, Kankia, et al. (2001) *J. Am. Chem. Soc.* 123(44):10799-10804, submitted herewith as **Exhibit F**, and Famulok et al. (2000) *Acc. Chem. Res.* 33(9):591-599, submitted herewith as **Exhibit G**. In contrast to the structure of dsDNA, Dr. LeClerc states that "the three-dimensional structure of the complex between [an] aptamer and [a] non-nucleic acid target differs fundamentally from the three-dimensional structure of a nucleic acid duplex molecule." (*Id.*, ¶6).

Due to the fundamental differences in the structure of the dsDNA hybrid, described in Ho, and aptamer/target G-quartet quadruplex complexes, as discussed above, Applicants respectfully submit that one skilled in the art would not have any expectation that polythiophenes could interact with aptamer/target sequences merely based upon their ability to interact with dsDNA sequences. According to Dr. LeClerc, prior to Applicants' invention, those skilled in the art recognized that it was "impossible to predict" and "even. . .considered unlikely" that polythiophenes would be capable of forming a complex with the quadruplex structure of the aptamer/target complex. (LeClerc Decl., ¶6). In addition to the unpredictability and likelihood that polythiophenes could even form a complex with the G-quartet quadruplex structures of aptamers/cognate targets, it was not expected and completely unpredictable whether such complex would produce an optical signal, *even if* it could associate with the aptamer/target structure. Dr. LeClerc testifies that "it was, at the timepoint of our invention, impossible to predict and had even to be considered as unlikely that polythiophenes as defined in [the above captioned] application can a) bind to said quadruplex structures and b) undergo a conformational change upon this binding which results in a detectable signal ... [T]he signal we obtained from the quadruplex structure differs from the signal obtain[ed] for a triplex structure." (*Id.*, emphasis added).

In view of the foregoing, Applicants respectfully submit that the skilled artisan, given the teachings of Ho and Gold, and particularly in light of the state of the art as evidenced by Exhibits A-G submitted herewith, would have no reasonable expectation of modifying the cited references in order to arrive at Applicants' presently claimed optical sensor. Because the detection of aptamer/target complexes was completely unpredictable and considered unlikely given the cited art and the state of the art as discussed herein, the references cannot support a *prima facie* case of obviousness under 35 U.S.C. § 103(a). Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection.

Rejection Under 35 U.S.C. § 103(a) – Michaud et al., Ho et al. McQuade et al., Gold et al. and Nilsson et al.

The Examiner has maintained the rejection of Claims 1, 4, 7, and 8 as allegedly being unpatenably obvious over Michaud et al. (2004) *Analytical Chemistry* 74:1015-20, McQuade et

al. (2000) *Chem. Rev.* 100:2537-3574, Gold, and Nilsson (2002) *J. Phys. Condens. Matter* 14:10011-10020 for the reasons set forth in the Office Actions mailed June 18, 2009 and October 7, 2008. In addition to the teachings of Ho and Gold discussed above, the Examiner relied upon Michaud providing the teachings that SEQ ID NO:3 is an adenosine-specific aptamer. The Examiner argued that McQuade teaches that "polythiophene antibody complexes are effective biosensors," and that Nilsson teaches that conjugated polythiophenes can be used to couple analyte-receptor interactions. The Examiner recognized that McQuade uses conjugated polythiophenes to transmit electrical (not optical) signals, and does not describe Applicants' polythiophene, but argues that the reference demonstrates "the versatility of polythiophenes" and lack of structural correlation between McQuade's conjugated polythiophene and the instantly claimed polythiophene "is not important in view of the teachings of Ho, et al, who teach the polythiophene recited in the claims." (Office Action, at 6). The Examiner concludes that one skilled in the art would recognize that the use of polythiophenes in an aptamer-based sensor, wherein SEQ ID NO:3 is the aptamer and adenosine is the target, would function.

Applicants respectfully disagree. For the reasons set forth above, the combined teachings of Ho and Gold cannot support a *prima facie* case of obviousness under 35 U.S.C. § 103(a). Applicants submit that the teachings of Michaud, McQuade and Nilsson do not cure the deficiencies in the *prima facie* case discussed above. Specifically, none of the references, either alone or in combination, provides a reason to expect that polythiophenes could associate with the particular G-quartet quadruplex structure characteristic of aptamer/target interactions. Nor do the references provide a reasonable expectation that even if such an association was possible, that it would result in an optically detectable signal.

As an initial matter, McQuade et al. are completely silent regarding the specific polythiophene derivatives required in Applicants' claims. Furthermore, the alleged "biosensor" depicted in Figure 24 of McQuade et al. is completely different from Applicants' claimed optical sensor. Figure 24 depicts a polythiophene conjugated to a platinum surface. (McQuade, p. 2567, Col. 2, 2nd paragraph). The polythiophene-coated platinum surface is contacted with magnetic beads functionalized with antibodies specific for atrizine, in the presence or absence of atrizine or atrizine-glucose kinase. After contact with atrizine, the capacitance current is measured. (McQuade, p. 2568 Col 1, 1st paragraph.) The polythiophene shown in Figure 24 is not sensor

that detects aptamer/target interactions. In fact, the polythiophene shown in Figure 24 is not a "polythiophene-antibody sensor" as suggested by the Examiner. Nor is the polythiophene shown in Figure 24 of McQuade even an "optical sensor," as there is no mention of optical measurement in connection with Figure 24.

Elsewhere on page 2567, McQuade et al. describe "antigens" labeled with polythiophene derivatives of unspecified structure. McQuade et al. report that upon binding a corresponding antibody, the optical density of the polymer band at 380 nm was observed to increase with increasing concentration of cognate antibody. McQuade suggests that the increase in polymer absorbance is due to "local pH change[s] that occur[] when the antigen and antibody bind." (McQuade, 2567, Col. 2). McQuade is completely silent about the use of an optical sensor that includes an oligonucleotide that binds to an aptamer. In particular, there is nothing to suggest that the polythiophene could associate with or detect the G-quartet quadruplex structure of an aptamer/target complex. As such, there is no scientific basis for the assertion that the skilled artisan, in view of the teachings of McQuade, would have a reasonable expectation of successfully arriving at Applicants' claimed optical sensor.

According to the Examiner, Nilsson teaches that "polythiophenes [in general] are sensitive to minor perturbations." The Examiner relies upon this single statement, as evidence that would suggest that Applicants' claimed optical sensors would work. As with McQuade et al., however, Nilsson is completely silent about the particular optical sensors recited in Applicants' claims. In fact, Nilsson relates to "amino-acid-functionalized polythiophene[s]." (Nilsson et al., Title). Further, as with McQuade, Nilsson provides no teaching that would lead the skilled artisan to reason that polythiophenes, and, in particular, Applicants' presently claimed polythiophenes, can be combined with single stranded oligonucleotide aptamers, to detect targets. Accordingly, Nilsson does not cure the deficiencies of the other cited references, in establishing a *prima facie* case of obviousness.

In view of the foregoing, Applicants respectfully submit that Michaud, Ho, McQuade, Gold, and Nilsson fail to provide the skilled artisan with a reasonable expectation of success, and therefore do not support a *prima facie* case of obviousness under 35 U.S.C. § 103(a). Applicants respectfully request reconsideration and withdrawal of the rejection accordingly.

No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, Applicant is not conceding in this application that previously pending claims are not patentable over the cited references. Rather, any alterations or characterizations are being made to facilitate expeditious prosecution of this application. Applicant reserves the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not reasonably infer that Applicant has made any disclaimers or disavowals of any subject matter supported by the present application.

CONCLUSION

In view of the above amendments and remarks, Applicants respectfully maintain that the claims are patentable and request that they be passed to issue. Applicants invite the Examiner to call the undersigned if any remaining issues may be resolved by telephone. Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: February 11, 2010

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EXHIBIT A

DECLARATION OF MARIO LECLERC

I, Mario Leclerc, do declare that:

1. I am a Canadian citizen residing at 909 rue Laudance, Apt. 316, Québec (Québec) G1X 5H7, Canada. I was professor of chemistry at the Université de Montreal between 1989 and 1998 and since 1998, I have been professor of chemistry at Université Laval.

2. Hoang-Anh HO, Maurice BOISSINOT and I are the inventors of EP 04737768 (in the following "the application") which is based on PCT application No. PCT/CA2004/000824 "Optical Sensor Based on Hybrid Aptamer / Conjugated Polymer Complexes" published under WO 2004/106544 on December 9, 2004. In this application, we describe and claim a system for the detection of various targets by using a sensor comprising an aptamer capable of binding to the target and a polythiophene. As presently claimed, these targets are different from nucleic acid molecules. In the following, I will explain why I am of the opinion that, at the time point of our invention, it could not have been expected that aptamer-polythiophene sensors are useful for the detection of non-nucleic acid targets.

3. The claimed sensors comprise two components, an aptamer and a polythiophene. Aptamers are synthetic nucleic acid molecules capable of binding to target molecules. At the time point of our invention, it was known that such aptamers can bind a variety of ligands including various metal ions, amino acids, drugs, proteins or other molecules (see application, [0003], last sentence and the literature cited therein). In fact, when we made our invention, it was common general knowledge how to identify such aptamers.

4. Furthermore, at the time point of our invention, the polythiophenes which form the second component of the claimed sensor were also generally known (see eg. our own invention WO 02/81735, D1 in the present proceedings).

5. Finally, at the time point of our invention, it was also known that it is possible to detect nucleic acid molecules in a solution by using a sensor comprising a nucleic acid molecule complementary to the nucleic acid molecules to be detected and a polythiophene. This was our invention which we described in D1. In the experiments leading to the invention described in D1, we could demonstrate that a polythiophene is capable of forming a complex with a double-stranded DNA. During formation of this complex, the polythiophene alters its structure which results in a detectable signal. This complex between double-stranded DNA and polythiophene is called "triplex" in D1 (see e.g. Figure 6 of D1).

6. However, it was not known and could not be expected at the time point when we made our invention that polythiophenes are capable of forming a complex with an aptamer binding a non-nucleic acid molecule. Even more, it could not be expected that the formation of such a complex results in a detectable signal. The reason for this is that, at the time point of our invention, it was known from the literature that the three-dimensional structure of the complex between the aptamer and the non-nucleic acid target differs fundamentally from the three-dimensional structure of a nucleic acid duplex molecule. As e.g. demonstrated in the attached publications (Nucleic Acid Selection and the Challenge of Combinatorial Chemistry; G-Quadruplex Formation of Thrombin-Binding Aptamer Detected by Electrospray Ionization Mass Spectrometry; Folding of the Thrombin Aptamer into a G-Quadruplex with Sr2+: Stability, Heat, and Hydration; A

DNA Aptamer as a New Target-Specific Chiral Selector for HPLC; Structure-Switching Signaling Aptamers; and Nucleic Acid Aptamers From Selection in Vitro to Applications in Vivo), aptamers can form G-quartets (often called quadruplex) upon the binding of a specific target. The formation of this structure has also been confirmed by ourselves in the experiments shown in the application (see e.g. Figures 1 and 3, page 11, [0041] and [00423]. Such G-quartets have a totally different conformational structure from double-stranded oligonucleotides or DNA. Due to the difference in the structure, it was, at the time point of our invention, impossible to predict and had even to be considered as unlikely that polythiophenes as defined in our application can a) bind to said quadruplex structures and b) undergo a conformational change upon this binding which results in a detectable signal. In this context, it is again important to note that the signal we obtained for the quadruplex structure differs from the signal obtain for a triplex structure in D1.

7. For this reason, it is my opinion that, at the time point when we made our invention, there was no hint in the literature that complexes between an aptamer and a non-nucleic acid target could be detected with the help of a polythiophene.

8. I declare that all statements made in this declaration of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6 April 2009
Date

Mario Leclerc

Mario Leclerc

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Research Achievements

- More than 160 publications in peer reviewed journals; cited over 6000 times; H-index of 45;
- 9 Invited book chapters;
- 9 Awarded or filed patents;
- 106 Invited lectures at prestigious institutions and meetings;
- Training of more than 32 graduate students;
- Invited Professor at the Université de Paris-XIII and Paris-VI, as well at the University of Durham.

Teaching Achievements

- Set up of new courses on Novel Materials at the undergraduate and graduate levels;
- Student evaluation of all courses above the Departmental average;
- 2006 « Professeur Étoile» Outstanding Teaching Award from the Faculty of Science and Engineering given to professors having superior student evaluation of their courses.

Academic Background

B.Sc. Chemistry	Université Laval	1983
Ph.D. Chemistry	Université Laval	1987
Postdoc	INRS-Énergie et Matériaux	1987-88
Postdoc	Max-Planck-Institute for Polymer Research	1988-89

Academic and Research Experience

Assistant Professor	Université de Montréal	1989-1994
Invited Professor	Université de Paris-XIII	May-June 1992
Associate Professor	Université de Montréal	1994-1998
Invited Professor	University of Durham	Aug.-Oct. 1996
Full Professor	Université de Montréal	1998
Full Professor	Université Laval	1998-
Holder of the Canada Research Chair (Tier 1) on	Electroactive and Photoactive Polymers	2001-
Invited Professor	Université de Paris-XIII	June-July 2001
Invited Professor	Université de Paris-VI	June-July 2003

Most significant work

1. H.A. Ho, M. Boissinot, M.G. Bergeron, G. Corbeil, K. Doré, D. Boudreau and M. Leclerc, «**Colorimetric and Fluorometric Detection of Nucleic Acids Using Cationic Polythiophene Derivatives**», *Angew. Chem. Int. Ed.*, 41, 1548-1551 (2002). Extensive studies over the last fifteen years on new chromic conjugated polymers and oligomers have led us to the rational design of novel optical and electrochemical biosensors. We have gained an international leadership in this field, as shown by many related invited lectures, invited reviews, and book chapters. Recent optimizations have led to the specific detection of less than 20 copies of genetic materials.
2. J.F. Morin and M. Leclerc, «**Syntheses of Conjugated Polymers Derived From *N*-alkyl-2,7-carbazoles**», *Macromolecules*, 34, 4680-4682 (2001). Our synthetic work on conjugated polymers has led us to prepare, for the first time, well-defined and conjugated poly(2,7-carbazole) derivatives. This novel and promising class of electroactive and photoactive polymers should open new research areas in the field of light-emitting diodes, thermoelectric devices, photovoltaic cells, and transistors. In particular, this novel class of conjugated polymers has allowed the development of light-emitting materials that cover the full visible range and this significant achievement has been outlined in chemistry.org (ACS), Heart Cut, October 28th 2002. We were invited to present our first work in this area at an ACS meeting (Boston, Fall 2002) and to write a review for Macromolecular Rapid Communications (2005).
3. M.Ranger, D. Rondeau, and M. Leclerc, «**New Well-Defined Poly(2,7-fluorene) Derivatives: Photoluminescence and Base-Doping**», *Macromolecules* 30, 7686-7691 (1997). For the first time, we have reported the synthesis of well-defined homo- and alternating fluorene-based conjugated polymers by Suzuki coupling reactions. This publication has paved the way to a new class of luminescent and conducting polymers. This important publication has already been cited 314 times. This pioneer work was followed by other studies from my group on luminescent and base-dopable conducting polyfluorenes. Our work on polyfluorenes has been the subject of an invited review in the Journal of Polymer Science, Chemistry Edition (2001).

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167.P.-L.T. Boudreault, N. Blouin, and M. Leclerc (Invited Review), " Poly(2,7-carbazole)s and related polymers", *Adv. Polym. Sci.*, 212, 99-124 (2008).

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165.H.A. Ho, A. Najari, and M. Leclerc (Invited Review), "Optical Detection of DNA and Proteins with Cationic Polythiophenes", *Acc. Chem. Res.*, 41, 168-178 (2008).

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Invited Lectures (106)

106. Cornell University, Department of Materials Science, *Conjugated Polymers: From Micro-Electronics to Genomics*, October 30th 2008, Ithaca, U.S.A..
105. Queen's University, Department of Chemistry, *Conjugated Polymers: From Micro-Electronics to Genomics*, October 3rd 2008, Kingston, Canada.
104. Entretiens Jacques Cartier 2008, *Piles solaires plastiques*, October 6th 2008, Montreal.
103. Gordon Research Conference, *Optical Detection of DNA and Proteins Based on Cationic Polythiophenes*, July 21st 2008, South Hadley, MA, U.S.A.
102. Macro-IUPAC 2008, *Biosensors Based on Cationic Polymers*, July 1st 2008, Taipee, Taiwan.
101. CSC Meeting 2008, MSED Award Lecture, *Conjugated Polymers : From Genomics to Micro-Electronics*, May 25th 2008, Edmonton, Canada.
100. ACFAS 2008, Colloque Biophotonique, *Polymères Conjugués: de la Micro-Électronique à la Bio-Photonique*, May 5th 2008, Québec.
99. TNO Holst Centre, *Conjugated Polymers: From Micro-Electronics to Genomics*, April 3rd 2008, Eindhoven, Holland
98. Europtrode IX, *Optical Detection of DNA and Proteins Based on Cationic Polythiophenes*, April 1st 2008, Dublin, Ireland.
97. Royal Society of Chemistry Meeting: Analytical Techniques for the Life Sciences, *Detection of DNA Damage From Radiation*, March 28th 2008, Dublin, Ireland
96. University of Windsor, Department of Chemistry, *Conjugated Polymers: From Micro-Electronics to Genomics*, January 25th 2008, Windsor, Canada.
95. Carnegie-Mellon University, Department of Chemistry, *Conjugated Polymers: From Micro-Electronics to Genomics*, October 9th 2007, Pittsburgh, U.S.A.
94. Université de Montréal, Department of Physics, *Conjugated Polymers: From Micro-Electronics to Genomics*, September 24th 2007, Montreal.
93. American Chemical Society Meeting, *Optical Detection of DNA and Proteins Based on Cationic Polythiophenes*, August 2007, Boston, U.S.A.
92. 2nd Organic & Nano Electronics Workshop, *Plastic Solar Cells Based on Poly(2,7-carbazole) Derivatives*, May 17th 2007, Montreal.

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90. Université Laval, Department of Physics, *Conjugated Polymers: From Micro-Electronics to Bio-Photonics*, February 27th 2007, Quebec City.
89. Université de Sherbrooke, Department of Chemistry, *Conjugated Polymers: From Micro-Electronics to Genomics*, February 21st 2007, Sherbrooke.
88. Konarka Inc., *Solar Cells Based on Poly(2.7-carbazole)s*, February 13th 2007, Lowell, U.S.A.
87. Université d'Angers, Laboratoire des Matériaux Moléculaires Organiques, *Ultra-Sensitive Optical Detection of DNA and Proteins From Conjugated Polymers*, November 23rd 2006, Angers, France.
86. International Conference on Synthetic Metals, *Ultra-Sensitive Optical Detection of DNA and Proteins From Conjugated Polymers*, July 4th 2006, Dublin, Ireland.
85. CSC 2006 Meeting, *Optical Detection of DNA by Conjugated Polymers*, June 1st 2006, Halifax, Canada.
84. University of Michigan at Ann Arbor, Department of Materials Science, *Ultra-Sensitive Optical Detection of DNA and Proteins From Conjugated Polymers*, March 10th 2006, Ann Arbor, U.S.A.
83. Université Laval, Department of Biochemistry, *Détection ultrasensible et spécifique d'ADN et de protéines: un défi de taille*, March 3rd 2006, Quebec City.
82. Université Laval, Faculty of Sciences and Engineering, *Plastiques, génétique et vidéo*, February 1st 2006, Quebec City.
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77. Université du Québec à Chicoutimi, Department of Chemistry, Biocapteurs optiques et électriques à base de polymères conjugués, October 22nd 2004, Chicoutimi.
76. Massachusetts Institute of Technology, Departments of Polymer Science and Chemistry, Optical and Electrochemical Biosensors Based on DNA/Polythiophene Complexes, October 13th 2004, Cambridge, USA.
75. Macro-IUPAC, Optical Sensors Based on Hybrid DNA / Conjugated Polymer Complexes, July 6th 2004, Paris, France.
74. Congrès Nano-Québec, Détection rapide et spécifique de l'ADN: un défi de taille, May 11th 2004, Montreal.
73. Second France-Canada Chemistry Congress, Plastics, Genetics, and Video, April 30th 2004, Nice, France
72. Georgia Tech, Department of Chemistry, Plastics, Genetics, and Video, April 1st 2004, Atlanta, USA
71. Steacie Institute for Molecular Sciences, NRC, Plastics, Genetics, and Video, March 9th 2004, Ottawa
70. Concordia University, Department of Chemistry, Plastics, Genetics, and Video, February 13th 2004, Montreal
69. IUPAC/CSC meeting, Chemical and Biochemical Sensors Based on Functionalized Polythiophenes, August 12th 2003, Ottawa.
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66. Université Paris VI, Polymères conjugués et génomique, June 30th 2003, Paris, France
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63. Université Paris VI, Polycarbazoles: Synthèse, Caractérisation et Applications, June 20th 2003, Paris, France.
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57. American Chemical Society Meeting, Novel DNA-chromic Conjugated Polymers, April 10th 2002, Orlando, U.S.A.
56. Steacie Institute for Molecular Sciences, Functional Polymers: From Electro-Optics to Genomics, April 5th 2002, Ottawa
55. Queen's University, Smart Materials Based on Functionalized Conjugated Polymers, December 5th 2001, Kingston.
54. Université de Montréal, Department of Physics, Electroactive and Photoactive Polymers, September 21st 2001, Montréal.
53. Boehringer-Ingelheim, Affinitychromic Polymers Applied to Combinatorial Chemistry, September 19th 2001, Laval.
52. 9th International Symposium of Macromolecular Metal Complexes, IUPAC-ACS, Ion-Responsive Conjugated Polymers, August 22nd 2001, New York.
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50. Université Paris VI, Diodes électroluminescentes polymères, July 3rd 2001, Paris, France.
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47. International Seminar on the Technology of Inherently Conducting Polymers: Smart Materials Based on Functionalized Conjugated Polymers, June 19th 2001, Niagara-on-the-Lake.
46. Canadian Electrochemical Society Symposium, Electrochemical Biosensors Based on Functionalized Conducting Polymers, June 1st 2001, Boucherville.
45. Congrès de l'ACFAS, Diodes organiques électroluminescentes polymères, May 15th 2001, Sherbrooke..
44. Centre Hospitalier Université Laval, Polymères synthétiques et séquences génétiques, April 27th 2001, Quebec City.
43. Materials Research Society, Electronic, Optical, and Optoelectronic Polymers and Oligomers, April 17th 2001, San Francisco, U.S.A.
42. Search for Electroactive Materials 2000, Novel Biosensors Based on Functionalized

Conjugated Polymers, December 2000, New-York, U.S.A.

41. Institut des Matériaux Industriels, Vers le Développement de Polymères Intelligents, October 2000, Boucherville, Canada
40. International Conference on Science and Technology of Synthetic Metals, ICSM 2000, Molecular Design of Chromic Functionalized Conjugated Polymers, July 2000, Bad Gastein, Autriche.
39. MACRO IUPAC 2000, Smart Polymers Based on Functionalized Conjugated Polymers, July 2000, Varsovie, Pologne
38. International Seminar on the Technology of Inherently Conducting Polymers: Smart Materials Based on Functionalized Conjugated Polymers: Present and Perspectives, June 2000, Napa, U.S.A.
37. Congrès de l'Institut de Chimie du Canada, Smart Materials Based on Functionalized Conjugated Polymers, May 2000, Calgary
36. XEROX Research Centre, Smart Materials Based on Functionalized Conjugated Polymers, March 2000, Mississauga.
35. Université Ottawa-Carleton, Smart Materials Based on Functionalized Polymers, March 2000, Ottawa.
34. International Seminar on the Technology of Inherently Conductive Polymers, Chromic Phenomena in Polythiophene Derivatives, September 1999, Toronto.
33. Centre d'Énergie Atomique, Synthèse et caractérisation de sels de polyfluorenyl-lithium, September 1999, Grenoble, France.
32. Journées Polymères Conducteurs '99, Vers le Développement de Biocapteurs Polymères (Keynote Speaker), September 1999, Aussois, France
31. 19th Annual Conference of the Canadian Biomaterials Society, Development of Photoactive and Electroactive Polymeric Biosensors (Keynote Speaker), June 1999, Québec
30. Congrès de l'Institut de Chimie du Canada, Field-Responsive Supramolecular Polythiophene Assemblies, June 1999, Toronto
29. Congrès de l'ACFAS, Etude de Polyesters Electroactifs et Photoactifs Dérivés d'Oligofluorènes, May 1999, Ottawa
28. Chemi Chromics USA '99, Chromic Phenomena in Polythiophene Derivatives, January 1999, New Orleans, U.S.A.
27. Université Laval, Vers le Développement de Polymères Intelligents, January 1999, Québec.
26. International Conference on Synthetic Metals '98. Development of New Base-Dopable Polymers. July 1998, Montpellier, France
25. Congrès de l'ACFAS. Synthèse et caractérisation de polyfluorènes régiorégulièrs pour applications en électro-optique, May 1998, Québec
24. Université Carleton. Electrical and Optical Properties of Novel Polythiophene Derivatives. March 1998, Ottawa
23. Conseil national de recherche du Canada. Electrical and Optical Properties of Novel Polythiophene Derivatives. December 1997, Ottawa
22. Congrès de l'Institut de chimie du Canada. Development of Novel Photochromic and Affinitychromic Polythiophene Derivatives. June 1997, Windsor.
21. Congrès GFP-SQP. Vers le développement de bio-senseurs. May 1997, Lyon, France
20. École Polytechnique Fédérale de Lausanne, Département des Matériaux. Electrical and Optical Properties of Polythiophene Derivatives. February 1997, Lausanne, Switzerland.
19. Institut National Polytechnique de Grenoble. Propriétés électriques et optiques de dérivés du polythiophène. Novembre 1996, Grenoble, France.
18. Institute Max-Planck for Polymer Research. Electrical Properties of Polythiophene

Derivatives. November 1996, Mainz, Allemagne.

17. Institute Max-Planck for Polymer Research. Chromism in Conjugated Polymers. November 1996, Mainz, Allemagne.
16. Xerox Research Centre of Canada. Optical Properties of Polythiophene Derivatives. February 1996, Mississauga.
15. IV International Conference on Advanced Materials. Chromism in Conjugated Polymers. August 1995, Cancun, Mexico.
14. Congrès de l'Institut de chimie du Canada. Self-Assembly in Chromic Polythiophene Derivatives. June 1995, Guelph.
13. ACS, 17th Biennal Symposium. Self-Assembly in Thermochromic Polythiophene Derivatives. November 1994, San Juan, Porto Rico.
12. Université du Québec à Montréal. Vers le développement de polymères intelligents. November 1994, Montréal
11. Max-Planck Institute Anniversary Meeting. Structure-Property Relationships in Thermochromic Polythiophene Derivatives. June 1994, Mainz, Allemagne
10. Université de Sherbrooke. Vers le développement de polymères intelligents. March 1994, Sherbrooke.
9. IBM Almaden Research Center. Synthesis and Characterization of Novel Electroactive and Photoactive Polythiophene Derivatives. March 1994, San Jose, USA.
8. 39th Canadian Spectroscopy Conference. Optical Properties of Poly(1,1,1,2,2,3,3,4,4,5,5,6,6-tridecafluorononylthiophene). August 1993, Québec.
7. IREQ (Hydro-Quebec) Electrical and Electrochemical Properties of Novel Conjugated Polymers. February 1993, Varennes.
6. Université de Paris-XIII, LRM. Propriétés Optiques de Dérivés du Polythiophène, May 1992, Paris, France
5. Université de Paris-XIII, LRM, Propriétés Electrochimiques de Dérivés de la Polyaniline, May 1992, Paris, France.
4. Congrès ICSM, Structural Effects on the Thermochromic Properties of Polythiophene Derivatives, August 1992, Goteborg, Sweden.
3. Université de Montréal, Département de chimie, Propriétés optiques et électriques de polymères conjugués, March 1992, Montreal.
2. Université Laval, Département de chimie, Propriétés optiques et électriques de polymères conjugués, January 1992, Quebec City.
1. McGill University, Department of Chemistry, Optical and Electrical Properties of Conjugated Polymers, January 1991, Montreal.

Other Evidence of Impact

- According to Science Citation Index, my papers have already received more than 6000 citations. I have a H-index of 45
- Macromolecular Science and Engineering Award 2008, Canada
- I am currently supervising 12 graduate students and 10 post-docs or research associates; and 32 students have already graduated from my laboratory. All of them have obtained a position in relation with their formation at the end of their studies.
- Member of the International Advisory Board of Macromolecular Chemistry and Physics, Macromolecular Rapid Communications, and Advanced Functional Materials (Wiley-VCH).
- Member of the Board for the Committee on Assays and Methods Development of the National Institute of Health (NIH) of USA, 2004.
- Reviewer for: Proceeding of the National Academy of Science (USA), Nature Materials, Journal of the American Chemical Society; Macromolecules; Chemistry of Materials; Advanced Materials; Advanced Functional Materials, Physical Review Letters; Journal of Organic Chemistry; Organic Letters; Macromolecular Chemistry and Physics; Macromolecular Rapid Communications; Journal of Physical Chemistry; Journal of Materials Chemistry; Thin Solid Films.

EXHIBIT B

CHEMICAL REVIEWS

Nucleic Acid Selection and the Challenge of Combinatorial Chemistry

Scott E. Osborne, and Andrew D. Ellington

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Nucleic Acid Selection and the Challenge of Combinatorial Chemistry

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Contents

I. Introduction

Drugs are small organic molecules. This axiom has dominated the thinking of both chemical academics and the pharmaceutical industry for decades, and for good reason: it works. Small organic compounds can be made relatively cheaply in large yields and have proven to be extremely effective at manipulating the biochemistry and physiology of humans. The effectiveness of small organic compounds is in turn due in large part to their ability to fit tightly into the active sites or regulatory domains of enzymes, the binding pockets of receptors, and the interfaces of macromolecular complexes.

Drugs can be designed or found or both. Designing new drugs generally relies on an intimate understanding of the biochemistry of a disease state, including in many cases a knowledge of the structure of a biopolymer target. In contrast, finding drugs involves screening compounds with assays that are indicative of a disease state. Until recently, the organic compounds that were screened were by and large natural products and their derivatives. However, synthetic chemists have more recently found that they can rival Nature's bounty by developing methodologies that allow the modular synthesis of compounds.[1,2] Multiple reagents of a given type (e.g., primary amines) are added either together or in parallel at different steps of a synthetic scheme, resulting in a "combinatorial" accumulation of compounds. As before, these combinatorial chemical libraries are screened using automated, high-throughput assays, and the best leads are subjected to further modification and testing.

While the current paradigm for drug development is the identification of small organic effectors of metabolism, it is not the only possible route. Biopolymers are also extremely adept at altering the function of organisms, organs, cells, or, ultimately, other biopolymers. The utility of biopolymer drugs is attested by the fact that proteins such as insulin and erythropoetin have generated markets that are in the billions of dollars. Biopolymer drugs can be identified by design or screening, but there is also an additional route to their discovery: selection. Random sequence mixtures of peptides, proteins, or nucleic acids can be generated and individual molecules winnowed from the population by allowing them to bind to or be utilized by an enzyme, receptor, or cellular target. The selected molecules are then amplified *in vitro* or *in vivo*. Multiple cycles of selection and amplification will foster competition between active compounds, and should eventually result in the purification of those few molecular species that have the highest affinity or efficacy for a given target (Figure 1). In contrast, most organic compounds are not readily replicable and therefore cannot be selected and evolve in the same way that biopolymers can. It should be noted, however, that organic chemists have begun to blur the distinction between replicable biopolymers and nonreplicable organic molecules. For example, chemical libraries can be prepared in which information regarding the binding activity or efficacy of an individual compound or set of compounds is linked to sequence (e.g., position scanning libraries or SURF),[3,4] locale (VLSIPS),[5] or an encoded replicable or non-replicable tag.[6–8] Each method allows sublibraries with a desired activity to be resynthesized and rescreened. Chemical libraries in effect become "replicable" and can be selected through the investment of organic synthetic effort rather than biosynthetic transformations.

In order for biopolymer selection to be recognized as an important contender in drug discovery efforts, especially in light of the great strides that have been made in combinatorial chemistry, one of two conditions must be recognized: either selected biopolymers must become drugs that have unique properties or are inherently better than small organics, or selected biopolymers must be useful for the generation of conventional pharmaceuticals. Using the *in vitro* selection of nucleic acids as an example, we seek to establish why both conditions are likely.

While there have been numerous reviews of both the methods for and the products of *in vitro* selection,[9–15] we will approach the subject from a more chemical perspective. A brief review of the

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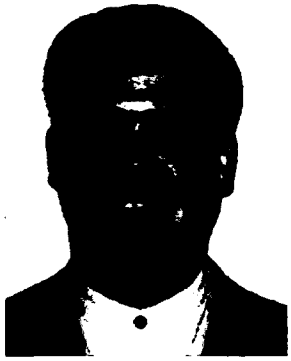


Scott E. Osborne was born in Lebanon, IN in 1968. While majoring in chemistry and lettering three years in collegiate golf at DePauw University in Greencastle, Indiana, he conducted independent research under Professor Bryan Hanson. After receiving his B.A. in 1990, he attended the University of Michigan to pursue a doctoral degree in chemistry. Under the supervision of Professor Gary D. Glick, he developed a method to conformationally restrain oligonucleotides using disulfide cross-links. His efforts in this area were recently recognized with the biennial presentation of the Kasimir Fajans award for outstanding dissertation. Since receiving his Ph.D. in 1996, Dr. Osborne has been performing postdoctoral research under the supervision of Professor Andrew D. Ellington. His research interest focuses on using the chemistry developed at Michigan to stabilize small oligonucleotides that bind specific ligands (minimal aptamers) as well as probe the conformations of unique nucleic acid:protein complexes. He enjoys spending his spare time with his wife, Amy, and son, Trevor, as well as working on his golf and bridge games.

Andrew D. Ellington was born in Independence, MO in 1959. He graduated from Michigan State University with a B.S. in Biochemistry in 1981, and from Harvard with a Ph.D. in Biochemistry and Molecular Biology in 1987. His graduate work with Professor Steven Benner explored the role of point mutations in protein evolution and was carried out both at Harvard and at the Eidgenosische Technische Hochschule in Zurich, Switzerland. During this time, he became interested in the early stages of molecular evolution and postulated interconnections between the RNA world hypothesis and intermediary metabolism. In order to test this hypothesis, he carried out postdoctoral research with Dr. Jack Szostak at Massachusetts General Hospital and developed a method for the selection of functional nucleic acids. As a faculty member in the Chemistry Department at Indiana University, he has expanded on these results and pursued interests ranging from origins of life chemistry to antiviral gene therapies. Minimal spare time is spent petting his dogs.

method will yield principles of experimental design that are generally relevant to combinatorial chemistry. The specificities and affinities of selected nucleic acids for their targets will be examined in order to determine if nucleic acids enjoy some special advantage over organic compounds. Conversely, we will recount how the two chief disadvantages of potential nucleic acid pharmaceuticals, size and instability, may be remedied by appropriate chemical modifications. Next, the implications of selection experiments for conventional drug design will be

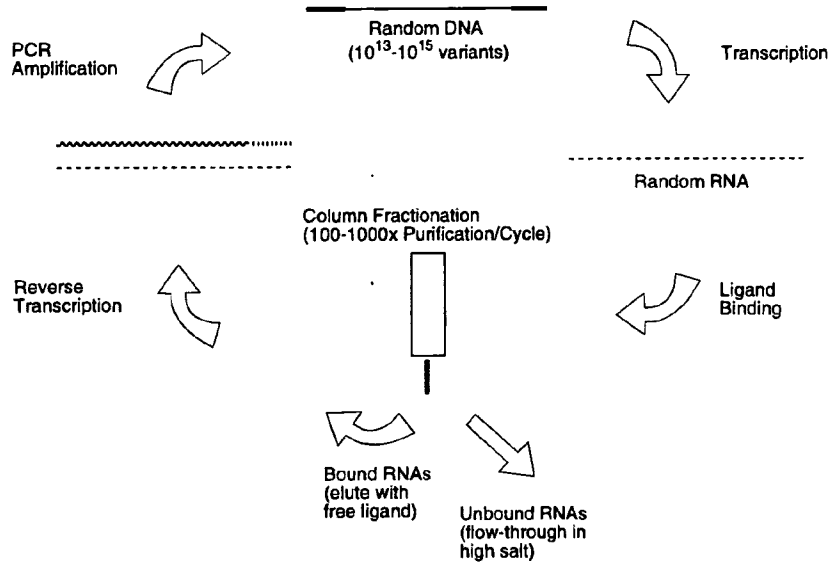


Figure 1. General schema for *in vitro* selection. A random sequence, single-stranded DNA pool is generated by chemical synthesis. Constant sequences flanking the random core are used for enzymatic manipulation. For example, the 5′-end might contain a T7 promoter and second strand priming site, while the 3′-end might contain a cDNA priming site (black boxes). Transcription with a RNA polymerase generates random sequence RNA, which can then be subjected to affinity purification (the selection process). Those RNAs that do not bind the immobilized ligand are washed away and discarded, whereas those which favorably interact with the ligand are selectively retained. Following affinity elution, the binding species are reverse transcribed to cDNA, amplified by PCR, and transcribed into RNA. The pool of random oligonucleotides is then once again subjected to the selection process. Multiple cycles of *in vitro* selection will result in selective purification of sequences that have a high affinity for the ligand of choice.

detailed. We argue that selection can be used both to define nucleic acid targets and to hone the specificities of compounds that bind to and inhibit the functions of nucleic acids.

II. A Brief History of Nucleic Acid Selection

It is frequently assumed and sometimes claimed on the basis of several arresting publications that the *in vitro* selection of nucleic acids was invented circa 1990. In fact, there is a large amount of prior art that precedes and presumes the development of modern methods for *in vitro* selection. The realization that nucleic acids could embody structure and function as well as carry information immediately suggested to experimentalists that it should be possible to carry out selection experiments at the molecular level. In the absence of a collection of purified, commercially available enzymes and amplification techniques such as the polymerase chain reaction (PCR), Sol Spiegelman and his co-workers used small RNAs derived from the Qβ bacteriophage genome and an encoded polymerase, the Qβ replicase, to develop a system for the *in vitro* replication and evolution of molecules.[16] This system accurately mimicked many of the properties of natural selection at the macroscopic level: the "fit" (or the fecund) survived, competition resulted in changes in the frequencies of different genotypes, mutants with novel properties emerged, parasites preyed on replicators.[17-19] Although the first *in vitro* selection experiments were performed over 20 years ago they nonetheless provided an important intellectual framework for what was to become a rapidly expanding field. The development of automated methods for the chemical synthesis of DNA allowed extremely diverse, random sequence libraries to be constructed, while the invention of *in vitro* amplification techniques, such as the polymerase chain reaction, enabled researchers to efficiently recover selected sequences and carry out multiple cycles of selection and amplification. Together, these advances prompted researchers to expand *in vitro* selection experiments beyond the molecular biology of the Qβ phage. A plethora of different selection experiments were carried out independently and nearly simultaneously, a fact which belies the notion that a single epiphany was responsible for the development of the field. Kevin Struhl, Larry Loeb, and their co-workers were among the first to take advantage of random synthetic libraries to probe oligonucleotide function, and selected functional promoter sequences either *in vitro* or *in vivo*.[20-23] As the use of random sequence libraries became increasingly popular, a number of researchers also defined binding sites for regulatory proteins.[24-28] The extrapolation of *in vitro* selection techniques from double-stranded to single-stranded nucleic acid shapes was obvious and led to the accelerated discovery of selected ligands (sometimes called aptamers) for a wide variety of targets.[29,30] Today, *in vitro* selection (sometimes called SELEX, the Systematic Evolution of Ligands by EXponential enrichment[29]) has moved beyond its academic roots to encompass the pursuit of lead compounds for therapeutic applications and the design of new diagnostic assays.

III. How It Works

A review of the methods used in *in vitro* selection experiments will provide essential background for a comparison with combinatorial chemistry. The *in vitro* selection process begins with a pool of sequence and structural diversity. Early experiments relied on the fact that Qβ replicase is extremely error-prone and generates numerous mutations each time it reproduces an RNA template. However, the number of different sequences that can be generated enzymatically is dwarfed by the number of different sequences that can be manufactured chemically. By programming a DNA synthesizer to add equal amounts of G, A, T, and C phosphoramidites at each step of a chemical synthesis, populations of 10^{13} to 10^{16} different DNA oligonucleotides can be routinely created. The use of solid phase oligonucleotide synthesis also allows researchers to more precisely control other characteristics of a nucleic acid pool. The length of the random sequence tract can be programmed to be anywhere from 1 to up to 140 or more residues. The degree of randomization can also be varied by varying the composition of the phosphoramidite mixtures. While many selections start with completely random nucleic acid pools, natural nucleic acid ligands can be partially randomized to contain, say, 70% wild-type and 10% of each non-wild-type residue (i.e., if a position in a ligand were a guanosine the pool would contain 70% G, 10% A, 10% T, and 10% C at that position). Partial randomization generates a pool with multiple mutations centered on the wild-type sequence.[31] Finally, chemical synthesis allows details regarding how a nucleic acid pool will be manipulated to be specified in advance. Constant sequence regions typically flank the random sequence tracts and allow the nascent DNA oligomer to be amplified by PCR. Promoter sequences included within the constant regions facilitate the enzymatic transformation of a DNA pool into an RNA or even modified RNA pool.

Each member of the nucleic acid pool has a different sequence and, thus, a different set of chemical groups that will fold into differing structures that have differing properties or are capable of different functions. Individual members of a pool are separated from one another on the basis of their ability to perform an arbitrary task. For example, RNA and DNA molecules that can carry out catalytic reactions such as ligation and phosphorylation have been culled from random sequence mixtures.[32,33] The focus of this review is nucleic acid binding species (aptamers), and a wide variety of methods have been adapted to their selection. For example, nucleic acids that interact with protein targets have been trapped on nitrocellulose filters or separated from uncomplexed species on native polyacrylamide gels. Aptamers that bind small molecules such as cofactors or vitamins can be isolated by affinity chromatography. Unbound nucleic acids are washed away from bound, and the bound species are then eluted by adding an excess of soluble ligand, changing solvent conditions, or cleaving from the solid support (via a reversible disulfide cross-link, for example). As was the case for pool synthesis, the progress of an *in vitro* selection can be more precisely controlled than the progress

of a natural selection. The stringency of a selection can be varied by changing the concentration of target, the buffer conditions for binding, and the volume of wash that precedes elution.[13,14,34]

Up to this point, there is little difference between screening a chemical library and a nucleic acid library. In both cases, only a few binding species may remain in the population following the initial screen. However, nucleic acids can be directly amplified using conventional molecular biology methods. In early experiments, Qβ replicase provided up to 10^9-fold amplification of successful sequences. Unfortunately, Qβ replicase discriminates between sequences on the basis of their suitability as templates or their replicability, and thus might inadvertently amplify a survivor with medium affinity at the expense of a poorer replicator with higher affinity. Newer methods allow greater control over the process of amplification and yield fewer artifacts. Selected DNA molecules can be readily amplified using PCR. RNA or modified RNA molecules must first be converted to DNA copies using reverse transcriptase, but can then also be amplified with the PCR. RNA can be regenerated from the amplified DNA via *in vitro* transcription with a bacteriophage polymerase, such as those from phage SP6 or phage T7. Other amplification methods combine these steps; for example, the transcription-based amplification system (TAS, also known as 3SR)[35] utilizes reverse transcriptase and RNA polymerase to cycle between DNA and RNA intermediates, mimicking the life cycle of retroviruses.

Multiple cycles of selection and amplification result in competition between binding species and allow aptamers with the highest affinity to be extracted from a population. The number of cycles of selection required to preferentially amplify a binding species or set of species is a function of the ligand, degree of randomization, and stringency, but from three to 15 cycles is typical. The entire process can take from a few days to a few months, but on average 2–4 weeks suffices. After the population has been pared down to those sequences which bind the ligand with high affinity, the pool is cloned and individuals sequenced. The number of discrete sequences that remain in a population following selection will range from one to thousands. Comparisons between selected sequences and their predicted secondary structures frequently reveal similar motifs or substructures that are important for binding function.[36] However, it is equally likely that aptamers will fall into unrelated families; that is, there may be multiple sequence "answers" to a given binding "problem".

IV. What Selection Reveals about Combinatorial Chemistry

Superficially, results garnered from *in vitro* selection experiments may not seem applicable to the design and screening of chemical libraries. As we have emphasized, chemical libraries are not replicable, while nucleic acids, for all of their diversity, are not small organic molecules that can be readily converted to drugs. However, nucleic acid libraries are chemical combinatorial libraries in the broadest sense of the term, and in both cases the problem of sorting through large numbers of different sequences (or compound formulae) and structures remains the same. Therefore, it may be possible to apply some of the lessons learned from nucleic acid selections to chemical library screens. For example, some of the considerations encountered during the design and execution of *in vitro* selection experiments, such as the problems of library coverage and skewing, may have counterparts in screens for pharmacophores. Such comparisons may or may not eventually be shown to be accurate, but their true value lies in how they can aid researchers to develop principles for experimental design in the nascent field of combinatorial chemistry.

Table 1. Number of Different Oligonucleotides Based on the Number of Random Residues

no. of random residues	no. of sequentially different compounds
1	4
4	256
10	$\approx 10^6$
20	$\approx 10^{12}$
30	$\approx 10^{18}$
40	$\approx 10^{24}$

Neither nucleic acid libraries nor chemical libraries completely span the universe of all possible structures. Because there are only four possible (natural) monomers, it is easier to quantitate the diversity and completeness of nucleic acid libraries (Table 1). For example, a nucleic acid pool that has 40 randomized positions potentially contains 4^{40} or about 10^{24} different sequences, each one of which will have a unique associated structure. In practice, though, synthetic limitations confine most libraries to at most 10^{16} different sequences. Therefore, in this example only one in every 10^8 possible base combinations is represented. Of course, mutations that arise during the amplification process increase the total number of species that are examined during the course of the selection, and for this reason amplification procedures that are inherently mutagenic are sometimes used. Nonetheless, for most selection experiments the important statistic is not the total number of species nor the coverage of "sequence space", but rather the number of successful species that are originally present in the actual population. This ratio varies from approximately $1{:}10^6$ to $1{:}10^{12}$, depending on the target and the stringency of selection. In general, library size is not an obstacle to the identification of aptamers. Similarly, although combinatorial chemical libraries are frequently only 10^3 to 10^6 strong, they nonetheless contain an impressive array of compounds, many of which are found to be active in a variety of assays. For example, Ellman has identified numerous compounds with novel binding specificities from a chemical library based solely on relatively limited modifications of a benzodiazepine backbone.[37]

It is less clear whether or not these incomplete nucleic acid libraries or chemical libraries of necessity contain the best possible compounds. Of course, in the absence of carrying out experiments with libraries that completely span sequence or structure spaces, it may never be possible to know. Once again, although, some *in vitro* selection experiments provide

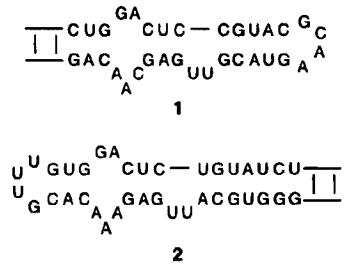


Figure 2. Consensus sequence of anti-Rev aptamers from nucleic acid libraries containing 18 (**1**)[38] and 32 (**2**)[39,40] random residues. In all cases throughout this review, the top stand of an oligonucleotide sequences reads 5′ to 3′.

clues as to whether incomplete libraries are "complete enough". RNA molecules that can bind to the Rev protein of HIV-1 have been selected from random sequence libraries of either 18 or 32 residues in length.[38-40] The library that contained 18 random sequence positions was likely complete, while the library that spanned 32 residues was not. Nonetheless, high-affinity aptamers with similar motifs were recovered from both libraries (**1** and **2**, Figure 2), indicating that at least in this instance the lack of coverage did not affect the outcome of the search. Similarly, aptamers that could bind to vascular endothelial growth factor (VegF) have been isolated from modified RNA libraries that spanned either 30 or 50 random sequence positions; the same high-affinity binding motif was recovered from both libraries.[41]

It may be possible to improve the probability of finding the best possible binding species by presenting random sequences within the context of a structured nucleic acid, just as antibodies present their variable loops within the immunoglobulin framework. For example, while Giver and co-workers isolated anti-Rev aptamers from a library in which up to 18 residues within an internal loop were randomized,[38] these authors also carried out a second selection starting from a library in which only 10 residues within an internal loop were randomized. The aptamers isolated from the two libraries had different sequences and binding characteristics: the aptamers isolated from the library with 18 randomized residues bound roughly 3-fold better than those isolated from the library with the shorter random sequence tract. These results can be interpreted to indicate either that more diverse libraries inherently contain better binding sequences or that the flanking constant sequences in the shorter library may have influenced the course of the selection. Similarly, Hamm synthesized nucleic acid libraries in which random sequence tracts were presented either in the context of a secondary structural elements, a G-quartet, or as an unstructured random sequence tract.[42] Aptamers that could bind to an antiferritin antibody were selected in parallel from both libraries. Unfortunately, the results of these experiments could not be interpreted to determine whether or not structure-specific libraries assist in identifying high-affinity binding species, since the primer binding sites in the constant regions paired with one another to form a strong secondary structure. This design resulted in the selection of species that could most efficiently bind primers and be amplified, rather than species that could bind to the antibody, and the selected populations were correspondingly skewed.

Overall, one possible interpretation of these experiments is that sequence motifs that are optimal for binding are frequent enough that they can be found even in incomplete libraries. Put another way, it may be either that long (and hence rare) sequence motifs are not uniquely suited to binding or that numerous variants of long sequence motifs (at least some of which will be found in an complete library) are more or less equally good at high-affinity binding. Translated from nucleic acid to chemical libraries, the implication is that complete coverage of functional space can be achieved even with limited libraries. Theoretical analyses by Peter Schuster and co-workers are consistent with these empirical observations.[43] These authors have demonstrated using a simple folding algorithm that even relatively limited sets of nucleic acid sequences can completely cover secondary structural space, making it unlikely that any but the rarest shapes will be underrepresented in a random sequence pool. However, there is an important caveat to this analysis: nucleic acids are linear polymers that fill shape and functional spaces by folding, and the folded structures have some inherent flexibility. Indeed, some selected nucleic acids, such as anti-nerve growth factor (NGF) aptamers,[44] only become structured on binding to their targets. Compounds within chemical libraries tend to be far more constrained, and hence may not represent the same dynamic ensemble of shapes. Instead, chemical libraries cover shape space by utilizing a much wider variety of fixed functional groups. Extending this comparison, it is possible that monomer diversity and monomer flexibility may compensate for one another in the design of chemical libraries: a smaller number of flexible monomers may be as useful for lead discovery as a larger number of more fixed monomers, despite the entropic cost that is paid on binding. This principle may explain why chemical libraries with some inherent flexibility, such as Chiron's peptoids, have proven successful.[45]

An alternative explanation for why selections that start with different pools give the same sequence answer is that the shortest answer typically wins. This phenomena has been called the "tyranny of short motifs",[46] and can be readily illustrated: in a random sequence pool, a particular 10 nucleotide motif will appear in roughly one in a million molecules, while a 20 nucleotide motif will appear in one in a trillion molecules, or a million-fold less frequently. Unless the 20 nucleotide motif has an affinity for a target molecule that is much higher than that of the 10 nucleotide motif, it will almost never be found in a selected population. While the inherent numerical advantage of shorter motifs can be partially offset by increasing the stringency of the selection, the highest affinity aptamers may never be known if small nucleic acid structures can form relatively tight complexes with their targets. The identification of short but suboptimal motifs has been observed in the selection of RNA molecules; several examples should suffice to link theory with experiment:

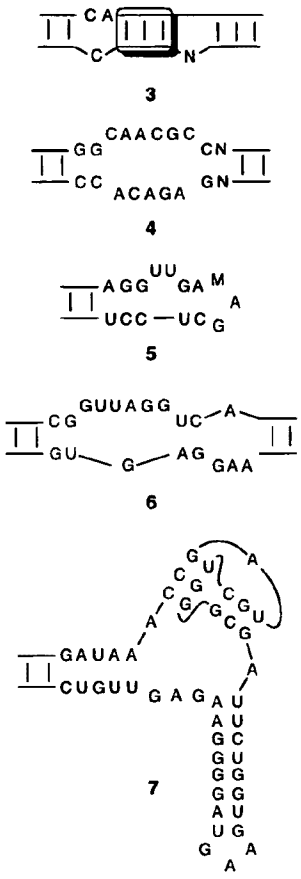


Figure 3. Consensus sequences of aptamers (3) that bind D-arginine nearly 2-fold better than L-arginine (box indicates that different canonical base pairs were conserved in 21 of 23 variants examined; N indicates any residue).[47] An aptamer was also recovered that could bind both arginine and guanosine (4). The aptamer found by Tao and Frankel (5) resembled a *TAR*-like structure (M indicates A, G, or U).[49] Famulok and co-workers derived two different aptamers (6)[50] and (7)[51] from long, random sequence pools.

(1) Selection of anti-arginine aptamers. Yarus and his co-workers have selected RNAs that can bind to arginine (3)[47] or to arginine and guanosine (4)[48] from random sequence pools (Figure 3). In both cases the random sequence regions were relatively short (25 residues), and the selected motifs utilized the constant flanking regions to form functional secondary structures; in other words, at least part of the information in the selected motif was "fixed" prior to selection. The dissociation constants for the arginine:RNA complexes ranged from 200 μM to 4 mM. Tao and Frankel also selected RNAs that could bind to arginine from a pool that spanned 30 random sequence positions.[49] While the recovered motifs (5) did not rely on constant sequences for binding function, the dissociation constants of the arginine:RNA complexes were again in the millimolar range. In contrast, Famulok selected anti-arginine aptamers (6) from a partially randomized pool that spanned 74 positions;[50] the dissociation constants for the arginine:RNA complexes were as low as 10 μM. The sequences of the selected aptamers bore no resemblance to those selected previously. This same group also selected anti-arginine aptamers from a completely randomized pool that spanned 113 positions;[51] the dissociation constants for the best arginine:RNA complexes were 330 nM. The sequences of the selected aptamers (7) bore no resemblance to those selected previously.

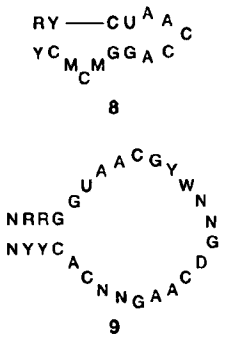


Figure 4. The two predominant aptamer families isolated from the selection against bFGF in the absence (8) and presence (9) of heparin (R = A or G; Y = C or U; M = A or C; W = A or U; D = A, G, or U; and N = any base).[52]

(2) A correlation between selection stringency and motif complexity was observed in selections that targeted basic fibroblast growth factor (bFGF).[52] Two RNA pools that spanned 30 random sequence positions were used as starting points for the isolation of anti-bFGF aptamers; the first pool was selected against bFGF alone, while the second pool was selected against bFGF in the presence of heparin. Two predominant aptamer families were isolated (Figure 4). Family 1 was predicted to form a stem-loop that contained a side bulge (8), while family 2 was predicted to form a stem capped by a very large loop (9). The first selection with the first pool returned both family 1 motif and family 2 motifs, while the second selection with the second pool returned primarily the family 2 motif. Both families interacted with the heparin binding site of bFGF, but family 2 aptamers bound roughly 10-fold better to bFGF than family 1 aptamers. While it may be that the differences in constant region sequences between the two pools favored the isolation of family 2 motifs in the second selection, the fact that this motif was found in several registers in selected aptamers argues against this. Rather, it may be that the inclusion of heparin favored the selection of family 2. This hypothesis was borne out by examining the complexity of the selected ligands. The "information content" of these families was estimated on the basis of the number of conserved and semiconserved residues. The family 1 motif was expected to occur once every 3.6×10^7 molecules, while the family 2 motif was expected to occur only once every 4.8×10^{11} molecules. Thus, the less stringent selection returned molecules of varying information content, while the more stringent selection returned the less probable motif. This is precisely the result that would have been predicted based on our understanding of the tyranny of short motifs.

(3) Ribozymes as well as aptamers appear to be subject to the "tyranny". Bartel and Szostak selected RNA ligases from a random sequence pool that

spanned 220 positions.[32] Three families of ligases were identified; two classes were structurally simple and contained multiple representatives, while the final class was extremely complex and contained only a single representative.[53,54] Again, this result is consonant with the tyranny of short motifs. Interestingly, the single, complex ribozyme should not have been found at all: its "information content" was so high that it should have been present only once in every 2.5×10^{18} sequences, while the original pool spanned only 1.4×10^{15} sequences. These results imply either that Bartel was extraordinarily lucky, or that multiple, complex motifs were present in the population. If the latter hypothesis is true, it may resolve a potential contradiction between the "tyranny" and the observation we made above that even incomplete libraries seem to be "complete enough" to successfully carry out selection experiments. While many complex, highly functional motifs may not be present in an incomplete library, if there are a number of these motifs at least one is likely to be found.

Taken together, these examples verify the reality of the "tyranny of short motifs," and further support the contention that longer, more complex pools can return aptamers that are as good as or better than those found in shorter, less complex pools, even when the latter are structurally constrained. In order to translate these results from RNA to chemical libraries, it is necessary to assume that the structural and functional diversity generated by sequence is equivalent to the structural and functional diversity generated by differing chemical groups. If this equivalence is true, the more structurally diverse the chemical library, the better the likelihood of selecting a high-affinity drug candidate. While this hypothesis currently forms the basis for the design of numerous chemical libraries, empirical support for it has been difficult to obtain.

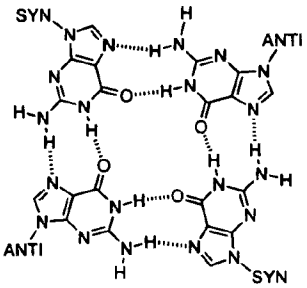
V. The Advantages of Selected Nucleic Acids

While organic compounds can interact tightly and specifically with target molecules, biopolymer ligands will frequently exhibit even better binding characteristics. This generalization is rooted in the fact that large "hosts" and large "guests" can form large interfaces. The best drugs will bind in clefts or crevices on a target molecule, forming an intricate network of interactions. The best biopolymers can also bind in clefts or crevices, but will in addition generate clefts or crevices of their own that can enfold surface features of the target. The more extensive interaction surface provides additional opportunities for a biopolymer "host" to discriminate between closely related target "guests". This generalization is also bolstered by the exquisite specificities of known antibody:antigen interactions. Consider that antibodies cannot only bind to surface features of protein antigens, but can actually induce conformational changes in the epitopes that are recognized. As the structure of the interface between antibody and antigen deforms, each molecule has several chances to reject any noncognate partner.

There are multiple examples of how selected nucleic acids can discriminate between even closely related targets. For example, aptamers selected to bind one reverse transcriptase (RT) do not interact with related proteins. RNA ligands to RT from the human immunodeficiency virus (HIV-1) did not inhibit similar enzymes from avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MMLV). RNA ligands to feline immunodeficiency virus RT did not inhibit similar enzymes from AMV, MMLV, and HIV-1.[55] Anti-bFGF aptamers bound to their cognate cytokine from 100- to 1000000-fold better than related fibroblast growth factors.[56] RNA aptamers directed against the βII isozyme of protein kinase C did not inhibit the α isozyme,[57] which is 80% homologous, and strongly discriminated against the βI isozyme, which differs by only 37 of 673 residues localized primarily in the carboxy terminus. DNA aptamers to human thrombin showed preferential binding to thrombin over other serine proteases.[58] The anti-thrombin aptamer could even discriminate against protein variants that differed by only a single amino acid. A mutation (R70E) in thrombin exosite I eliminated interactions with the thrombin aptamer.[59] Interestingly, DNAs selected to bind to the R70E variant could interact with wild-type thrombin as well,[60] indicating that there may be aptamer "generalists" as well as "specialists".

As expected, the basis for aptamer selectivities appears to be their ability to meld with surfaces features of a protein, fitting to crevices and/or enfolding protrusions. Aptamers selected to bind to the human T-cell leukemia virus regulatory protein Tax interacted with a particular subset of functional features, blocking protein:protein interactions with some transcription factors but not others.[61] Aptamers selected to bind to $Q\beta$ replicase formed two classes that did not compete with one another for binding and recognized independent sites on the protein.[62] The interaction of aptamers with particular protein surface features has been impressively validated by structural studies of the anti-thrombin aptamer. The DNA 15-mer is found to form a G-quartet stack similar to that observed for telomeric DNA (Figure 5).[63-66] Mutational and chemical protection analysis had indicated that the aptamer interacted with the anion-binding exosite of thrombin,[59,67] and this was confirmed by a crystallographic study.[68] A loop on the thrombin aptamer pokes into the exosite, and phosphate oxygens contact basic amino acids while thymines are involved in hydrophobic interactions.

Aptamers can also wrap around amino acid sequence tracts, similar to the way antibodies bind to continuous epitopes on proteins. Nieuwlandt and coworkers selected aptamers that could bind to a peptide, substance P.[69] The aptamers could efficiently recognize oligopeptides as short as 7 amino acids in length, but did not recognize the sequence presented in a reverse orientation. Xu and Ellington selected aptamers that could bind to a 17-mer α-helical peptide derived from the Rev protein.[70] Binding studies with peptide variants indicated that some aptamers recognized individual amino acids. The aptamers could also recognize the same amino acid sequence when presented within the context of the Rev protein, just as antipeptide antibodies can frequently cross-recognize proteins.



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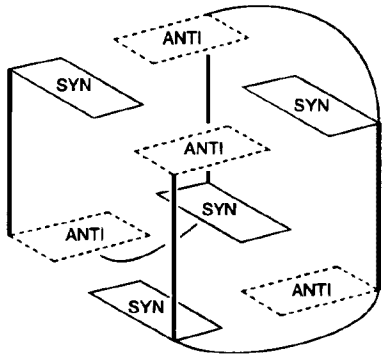

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Figure 5. Base-pairing scheme of G-quartet (**10**) in which the bases alternate between a *syn* and *anti* torsion angle in order to form a web of hydrogen bonds. Base-stacking arrangement of G-quartets found in the anti-thrombin aptamer (**11**) also alternate from *syn* to *anti*.[68]

The affinities of selected nucleic acids for their targets are generally quite good, with dissociation constants falling in the subnanomolar to decananomolar range. These values are comparable to those of conventional drugs and of some monoclonal antibodies or Fabs. The relative binding abilities of aptamers have previously been reviewed (Table 2).[9,12]

Recently, several methods have been developed to enhance the affinities of selected nucleic acids for their targets. Small molecules have been appended to aptamers in order to direct them to bind at particular sites on a target. In a variation of this method, Lin and co-workers tethered an aptamer selected to bind human neutrophil elastase to a tetrapeptide that was a weak inhibitor of the enzyme.[71] The two compounds bound at separate sites on the enzyme surface, and thus could act cooperatively when linked together. The affinity of the aptamer for elastase was improved by a modest 2-fold ($K_D \approx 7$ nM), but the inhibitory ability of the peptide increased by five orders of magnitude. In a separate set of studies, Smith and co-workers conjugated a RNA pool to a suicide inhibitor of elastase and selected RNA conjugates that could become covalently bound to the target.[72] Again, the small molecule increased the (noncovalent) binding affinity of the aptamer by around 20-fold. More impressively, conjugation reduced cross-reactivity with related targets by over 100-fold. Nucleic acids that can

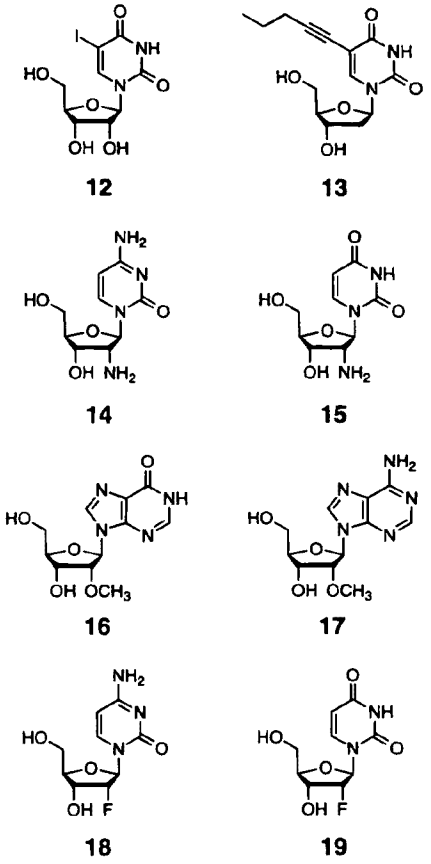
Table 2. Relative Binding Abilities of Aptamers to Various Proteins

ligand	aptamer motif	conjugate ligand K_D (nM)	ref
Nucleic Acid Binding Proteins			
AMV RT	hairpin	2	83
ASF/SF2, SC35	hairpin	N/A[a]	155
E. coli 30S Particles + S1	pseudoknot	5	156
E. coli 30S Particles - S1	Shine-Dalgarno	7	156
E. coli rho Factor	hairpin	1	*b*
E. coli S1 Protein	pseudoknot	4	156
FIV RT	hairpin	2	55
Hel-N1	U-rich	N/A	*c*
HIV-1 Int	complex	20	*d*
HIV-1 Rev	bulge	1	38–40
HIV-1 RT	pseudoknot	5	83
HIV-1 Tat	hairpin/bulge	5	39
HTLV-1 Rex	bulge	N/A	147
MMLV RT	hairpin	10	83
$Q\beta$ replicase	pseudoknot	5	62
R17 coat protein	hairpin	5	*e*
ribosomal protein L22	hairpin	N/A	*f*
T4 DNA polymerase	hairpin	5	29
U1A	hairpin	5	*g*
U2AF	single strand	1	154
Non-Nucleic Acid Binding proteins			
anti-gp10 antibodies	hairpin	N/A	*h*
anti-insulin receptor Ab	hairpin/bulge	2	*i*
bFGF	hairpin	0.2	52
HTLV-1 Tax	hairpin	100	61
NGF	pseudoknot	100	44
PKC bII	complex	7	57
substance P	hairpin/bulge	190	69
thrombin	hairpin	9	157
VegF	hairpin/bulge	0.2	126

[a] Not available or determined. [b] Schneider, D.; Gold, L.; Platt, T. *FASEB J.* **1993**, *7*, 201. [c] Levine, T. D.; Gao, F.; King, P. H.; Andrews, L. G.; Keene, J. D. *Mol. Cell. Biol.* **1993**, *13*, 3494. [d] Allen, P.; Worland, S.; Gold, L. *Virology* **1995**, *209*, 327. [e] Schneider, D.; Tuerk, C.; Gold, L. *J. Mol. Biol.* **1992**, *228*, 862. [f] Dobbelstein, M.; Shenk, T. *J. Virol.* **1995**, *69*, 8027. [g] Tsai, D. E.; Harper, D. S.; Keene, J. D. *Nucleic Acids Res.* **1991**, *19*, 4931. [h] Tsai, D. E.; Kenan, D. J.; Keene, J. D. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 8864. [i] Doudna, J. A.; Cech, T. R.; Sullenger, B. A. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 2355.

covalently cross-link to their targets can also be selected on the basis of chemistry inherent in the nucleic acid, rather than on the chemistry inherent in the target or its active site.[73] A nucleic acid pool was synthesized with a photolabile nucleotide, 5-iodouridine (**12**), in place of uridine, mixed with a protein target, HIV-1 Rev, and then exposed to UV light (Chart 1). Those nucleic acid species that bound Rev and positioned the photolabile nucleotide adjacent to a chemically compatible surface feature of the protein became cross-linked. The covalently bound species were isolated, amplified, and further selected both for their ability to bind in the absence of irradiation and for their ability to form extremely tight complexes that cannot be dissociated by denaturation. The ligands that were eventually discovered could bind with subnanomolar affinities and efficiently cross-linked to the target protein. Amazingly, some of the selected species also had the ability to cross-link even in the absence of irradiation. One of the many nucleophiles on the surface of the protein likely catalyzed the displacement of iodine and the formation of a covalent bond to the 5 position of uridine.

Chart 1. Structure of Modified Nucleotides Used To Increase Nuclease Resistance and Chemical Stability of Aptamers



VI. Making Nucleic Acids More Stable and Smaller

Any advantages selected nucleic acids may have in terms of affinity and specificity are obviated if they are too fragile for clinical use or too expensive to make. Unfortunately, RNA can be quickly degraded by a wide variety of nucleases found in sera and cells. In fact, the half-life of most natural or selected RNA molecules in sera is minutes or less. DNA molecules are slightly more stable, with half-lives up to hours. In addition, the development of antisense technology has collaterally yielded a variety of methods for the large scale synthesis and *in vivo* stabilization of DNAs. Thus, it is important to realize that *in vitro* selection experiments can be carried out with DNA libraries as well as with RNA libraries (Table 3). The first example, already described, of a DNA selection against a protein target was the identification of anti-thrombin aptamers.[58] While the initial selections returned a small, readily manipulable 15-mer motif, additional selections have found that this motif can be expanded to include an adjacent stem structure.[74] Anti-IgE aptamers were selected from a random sequence DNA pool that spanned 40 or 60 positions.[178] The aptamers bound their target with an affinity of 10 nM. Anti-reverse transcriptase aptamers have been selected from a random sequence DNA pool, bound the enzyme with a K_D of about 2 nM, and inhibited reverse transcriptase activity with a K_i as low as 0.3 nM.[75] As was the case with aptamers selected from RNA libraries, the selected DNA was specific for its cognate reverse transcriptase.

Table 3. Aptamer Selections Carried out Using DNA Pools

ligand	pool	aptamer motif	conjugate ligand K_D (nM)	ref
E. coli metJ protein	dsDNA	unknown	1	*a*
elastase	ssDNA	G-quartet	7	71
HIV-1 RT	ssDNA	stem/bulge	2	75
IgE	ssDNA	stem/bulge	10	178
Lrp	dsDNA	duplex	2	*b*
thrombin	ssDNA	G-quartet	25	58

[a] He, Y.; Stockley, P. G.; Gold, L. *J. Mol. Biol.* **1996**, *255*, 55. [b] Cui, Y.; Wang, Q.; Stormo, G. D.; Calvo, J. M. *J. Bacteriology* **1995**, *177*, 4872.

Table 4. Aptamer Selections Carried out Using Modified DNA or Modified RNA Pools

ligand	modified bases in pool[a]	aptamer motif	conjugate ligand K_D (nM)	ref
bFGF	**14–15**	unknown	0.3	78
elastase	**14–15**	G-quartet	15	77
HIV-1 Rev	**12**	hairpin/bulge	1	73
IgE	**14–15**	G-quartet	30	178
RSV	**18–19**	various	3 μg[b]	81
thrombin	**13**	hairpin	400	76
VegF	**14–17**	hairpin	0.3	41

[a] See Chart 1 for structures. [b] Expressed in terms of total viral protein concetration instead of K_D.

A. Modified Nucleotides

Further stabilization can be afforded by the introduction of chemical modifications into selected nucleic acids (Table 4). In general, modifications of the 2′ position of ribotides or of the phosphodiester backbone interfere with the enzymatic mechanisms of nucleases and lead to concomitant increases in stability. Modified nucleotides can be introduced either into the initial pool (presubstitution) or following selection of binding species (postsubstitution) or both.

Presubstitution strategies rely on the ability of DNA polymerases, or RNA polymerases and reverse transcriptases, to utilize modified nucleotides as substrates.[76,77] Several selections have now been carried out in which one or more of the canonical nucleotides in the original library was replaced with a modified nucleotide (Chart 1). In the first reported use of modified bases during selection anti-thrombin aptamers were isolated from a DNA pool that contained 5-(1-pentynyl)-2′-deoxyuridine (**13**).[76] This modified nucleotide had previously been shown to enhance the activities of antisense oligonucleotides and could be readily incorporated into growing strands by a thermostable polymerase. The anti-thrombin aptamers were dependent on the modified base for activity, but actually had less affinity for thrombin than aptamers selected from a "natural" DNA library. The modified nucleotides 2′-aminocytidine (**14**) and 2′-aminouridine (**15**) interfere with ribonuclease cleav-

age, are recognized by T7 RNA polymerase and AMV reverse transcriptase, and have been substituted for their natural counterparts in a number of selections. Lin and co-workers selected nucleic acid ligands that could bind to human neutrophil elastase and were completely substituted with these modified pyrimidines.[77] The aptamers were dependent on the amino sugars for high-affinity binding, and the half-lives of the substituted RNAs were increased to many hours in serum. Similarly, aptamers that bound bFGF or vascular endothelial growth factor (VegF) were selected from pools containing modified pyrimidines.[41,78] The anti-bFGF aptamers had K_D values as low as 3.5×10^{-10} M and their stability in serum was increased by at least 1000-fold relative to unsubstituted RNAs (other experiments suggest that the relative stabilities of such substituted RNAs is likely increased by 10^6-fold[79]). Sequence and deletion analyses of the anti-VegF aptamers allowed a minimal binding species (32 residues) to be designed. In order to further stabilize the construct to exonuclease degradation, several residues containing phosphorothioates were added to the 5′- and 3′-termini. The minimal aptamer containing modified nucleotides and phosphorothioate caps was found to form a complex with VegF that had a dissociation constant of 2.4 nM and its half-life in rat urine (an even more hostile environment than serum[41]) was 17 h.

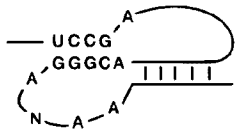
Because the identity of individual residues in selected sequences is critical for binding it would not necessarily be predicted that postsubstitution of modified nucleotides would lead to ligands that were both stable and efficacious. Surprisingly, modified nucleotides can be used to improve both properties. The anti-VegF aptamers that contained 2′-aminopyrimidines were further substituted with 2′-*O*-methylpurines (**16** and **17**).[41] Complete substitution of all purines in the minimal motif led to a drastic (100-fold) decrease in binding affinity. However, selected substitution of 10 of the 14 purines present actually led to a 10-fold increase in binding affinity and an 8-fold increase in stability; the final aptamer had a half-life in urine of 131 h. The improvement in affinity was largely due to a decrease in the dissociation rate of the complex. Green and co-workers were also able to use to postsubstitution to radically improve the stability of aptamers selected from RNA pools.[80] A novel interference analysis was used to determine which residues in an anti-reverse transcriptase aptamer could be substituted with 2′-*O*-methyl nucleotides. The aptamer was synthesized with a 1:1 mixture of normal nucleotides and 2′-*O*-methyl nucleotides, the population was sieved based on the basis of its ability to bind to HIV-1 RT, the bound species were hydrolyzed with base, and the mixture was analyzed by gel electrophoresis. When the incorporation of a 2′-*O*-methyl nucleotide at a given position interfered with binding, the selected population would contain predominantly normal ribotides at that position. Such "critical" positions were identified on the basis of their increased susceptibility to alkaline cleavage. Only two positions were found to require a 2′-hydroxyl moiety for high-affinity interactions with reverse transcriptase, the remainder of the ligand could be completely substituted with 2′-*O*-methyl nucleotides. Selection can also be used to facilitate postincorporation of modified nucleotides. Aptamers that could bind to Rous sarcoma virus (RSV) particles were selected from a random sequence RNA pool.[81] When aptamers from the selected pool were completely substituted with 2′-fluoropyrimidines (**18** and **19**), much of their binding activity was lost. Therefore, the selected pool was substituted with modified nucleotides and further selected for an additional three cycles for binding to RSV. The final pool containing modified pyrimidines could interfere with viral replication almost as well as the unmodified, parental population, indicating either that sequences that were dependent on unsubstituted pyrimidine nucleotides were eliminated from the population, or that aptamers containing positions that required unsubstituted pyrimidine nucleotides had mutated.

Given the chemical differences between RNA, modified RNA, and DNA it is perhaps not surprising that selections that start with different pools return different aptamers. Anti-RT aptamers selected from RNA pools have different sequences and structures than anti-RT aptamers selected from DNA pools; the same is true for anti-thrombin aptamers and for anti-ATP aptamers.[82] Similarly, anti-FGF and anti-VegF aptamers selected from RNA pools have different sequences and structures than the corresponding aptamers selected from modified RNA pools containing 2′-aminopyrimidines (**14** and **15**). Finally, anti-elastase aptamers selected from DNA pools have different sequences and structures than the corresponding aptamers selected from RNA pools containing 2′-aminopyrimidines or conjugated to a suicide inhibitor. These results may appear inconsistent with the fact that postsubstitution strategies for stabilization are successful. If most of the residues and the structural context of a selected sequence motif are not highly dependent on either 2′-hydroxyl or 2′-amino moieties, then the same motif might be expected to be selected regardless of the chemical nature of the starting population. In this respect, it should be noted that the anti-elastase aptamers selected from a modified RNA pool have some residual binding activity when the sequence is composed solely of ribotides.[77] Thus, while it is likely that DNA and RNA of the same sequence assume quite different structures, the RNA and modified RNA of the same sequence may be more structurally similar.

B. Conformationally Restraining Minimal Oligonucleotides

Although modified aptamers can resist degradation, their size (generally greater than 30 nucleotides in length) may remain a barrier to cost-effective synthesis. Individual residues in an aptamer either directly contact small molecule or protein targets or else provide a structural context for those residues that do make direct contact. An easy way to observe this is to examine how the functional residues and secondary structures were determined for an anti-RT aptamer.[83] The original selection produced a series of ligands which contained a conserved se-

quence motif; this motif could be folded into a pseudoknot structure (**20**). The motif was synthe-



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sized as a "doped" sequence pool and functional variants were selected. Sequence analysis of the selected species demonstrated that some residues could not vary without loss of binding function; these presumably directly contacted the protein or precisely positioned other chemical moieties to contact the protein. Other residues covaried in a manner that was consistent with the formation of a Watson–Crick paired stem. While the identity of these residues was relatively unimportant for function, the structural scaffold they formed was important for function. Overall, the selection experiments proved that a short (27-residue) RNA pseudoknot was sufficient for high-affinity interactions with HIV-1 RT.

Chemical linkers can be used to reduce the size of scaffolding elements (and the overall cost of synthesis) while retaining the positions of functional residues and moieties. A range of chemical and enzymatic methods are available to incorporate interstrand cross-links into the helical regions of DNA and RNA.[84] Alternatively, nucleotide loops have been used to "cap" functional nucleic acids at one (hairpin, **21**) or both (double-hairpin or "dumbbell", **22**) ends of a helix (Figure 6).[85–88] Certain loop sequences, such as GCGAAGC in DNA[89] and UUCG in RNA,[90]

21

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23

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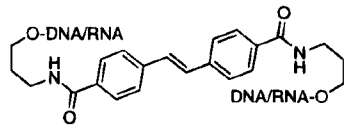
Figure 6. Schema of hairpin and dumbbell structures containing either nucleotide or non-nucleotide linkers. Double-hairpins can be made synthesizing an oligonucleotide in which the termini are aligned by a complementary strand and abut one another, enzymatically phosphorylating the oligonucleotide with T4 polynucleotide kinase, and ligating with DNA or RNA ligase. Alternatively, dumbbells can be made chemically by phosphorylating during solid-phase synthesis and ligating using either 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC)[87] or CNBr.[88] Yields are typically in the 30–50% range, however, there have been examples of yields as high as 70%.

have been found to be particularly effective at stabilizing structure and imparting nuclease resistance. Non-nucleotide linkers, such as (poly)ethylene glycol (PEG), have also been used to cap helices at one (**23**) or both (**24**) ends of the helix.[91] These reagents are easier and cheaper to make, and are commercially available. Structures constrained by PEG caps have been used to probe nucleic acid: protein interactions.[92] In addition, Kool and co-workers have shown that PEG linkers can increase the stability of oligonucleotides toward exonucleases.[93] Similarly, Lestinger has shown that a stilbenedicarboxamide linker (**25**) can increase the stability of an oligonucleotide.[94] The stilbene linker is rigid



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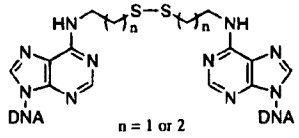
enough to effectively constrain the terminus of the helix, but also stacks with and stabilizes the helix.

These methods, however, have some potentially serious drawbacks. First, in some cases the linkers can distort or alter native helical geometry. Second, construction of dumbbell oligonucleotides can be technically difficult and in many cases poor overall yields are obtained. Finally, chemical linkers frequently span the terminal 3′- and 5′-hydroxyls of an oligonucleotide and interfere with enzymatic radiolabeling of the sample for biochemical assays such as footprinting and sequencing.

With knowledge of these drawbacks, a more recent technique to constrain oligonucleotides has emerged. Reactive functional groups have been engineered into oligonucleotides so that they are in close proximity with one another in the final structure and thus specifically react to form cross-links. Of these, thiols have been the reactive functional group of choice because: (1) disulfide bonds are formed in high yield, often quantitatively;[95] (2) the mild redox chemistry of thiol–disulfide interconversion provides little potential for undesired side reactions;[96] (3) disulfides are stable to a wide variety of solvents and reagents;[97,98] and (4) engineering specific conformations using disulfide cross-links is well-precedented in the peptide and protein literature.[99,100]

Lipsett first reported the formation of disulfide cross-links in a nucleic acid in 1966.[101–103] In those experiments, she found that $tRNA^{Tyr}$ from *E. coli* that naturally contained 4-thiouridine residues could be oxidized with iodine to form an intramolecular disulfide cross-link. Unfortunately, the potential utility of disulfide cross-links for studying nucleic acid structure was not realized because other, critical analytical methods such as RNA sequencing were not available. Therefore, it was not until 1991 that Glick and Verdine independently reported that disulfide cross-links could be site-specifically introduced into oligonucleotides via solid-phase chemical synthesis.

Verdine and co-workers have explored methods for introducing disulfide cross-links into the helical regions of nucleic acids.[104,105] In their method, thio-



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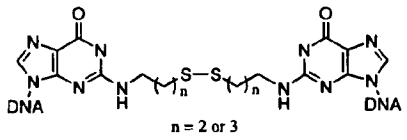

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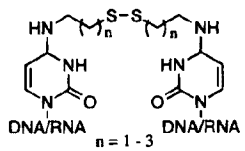

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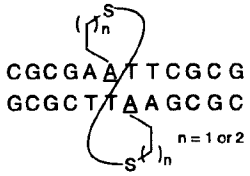

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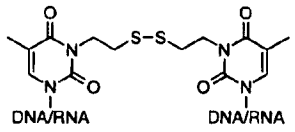
Figure 7. Thiol-modified nucleosides used by Verdine and co-workers to conformationally constrain oligonucleotides with disulfide cross-links (**26**–**28**).[104] These disulfide cross-links are located within the grooves of the helix and can be introduced at consecutive base pairs. The oligonucleotide shown in **29** is an example of how a disulfide can be introduced across the major groove of the helix.

alkyl tethers of varying lengths (3- to 5-atoms) are positioned at the N^6 of adenosine (**26**),[105] the N^2 of guanosine (**27**),[106] or the N^4 of cytosine (**28**).[107,108] The resultant disulfides occupy the grooves of the helix (Figure 7). In their first report, thiol-modified adenosines were placed at consecutive base pairs on opposite strands of a duplex (**29**) and under oxidative conditions formed an interstrand disulfide cross-link in the major groove of the helix. These cross-links imparted increased thermal stability (ΔT_m = 19 °C) to the duplex relative to the unmodified sequence and resulted in only minimal distortion of its native geometry. Since this initial report, Verdine and co-workers have used disulfide chemistry to trap four different oligonucleotide conformations: a bent duplex,[109] a minor groove cross-link,[106] a torsionally strained duplex,[107] and a RNA hairpin.[108]

An alternative means to stabilize nucleic acids via disulfide cross-links has been described by Glick and co-workers.[110,111] In their work, alkyl mercaptan groups are appended to bases (**30** and **31**) or sugars (**32**) to form disulfide cross-links at specific sites (Figure 8). In contrast to positioning disulfide bonds



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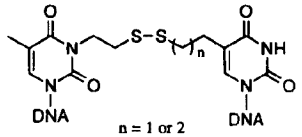

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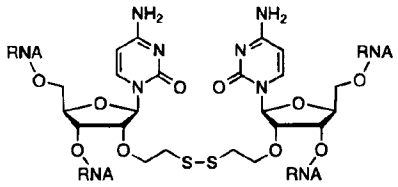

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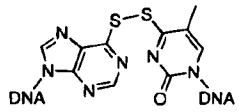
Figure 8. Thiol-modified nucleosides used by Glick and co-workers to conformationally constrain oligonucleotides with disulfide cross-links (**30**–**32**).[111] Disulfide cross-link formation is designed to occupy sterically accessible spaces and therefore to be structurally nonperturbing. For example, a duplex can be stabilized (**33**) by placing N^3-(thioethyl)thymidines (**30**) at the termini so that the cross-link resides at the ends of the helix.[117]

in the grooves of helices, these cross-links occupy sterically accessible spaces and therefore should conformationally stabilize nucleic acids without significant perturbation of their native structures. Using this chemistry, disulfide bonds have been introduced into nucleic acid secondary and tertiary structures such as hairpins[110,112] and duplexes,[111] triple helices,[113] and tRNA.[114] The structural[115,116] and thermodynamic[117,118] properties of these cross-linked oligonucleotides have been fully described. For example, when the terminal bases of the DNA dodecamer d(CGCGAATTCGCG)$_2$ (**33**) were replaced with N^3-(thioethyl)thymidines (**29**), the strands were prevented from dissociating and the thermal stability of the duplex was dramatically increased (ΔT_m = 38 °C).[111] Thermodynamic analysis (UV and DSC) reveals that the enhanced stability is entropic in origin and structural analysis (NMR and CD) indicates that the helical structure has not been compromised.[117] In addition, preliminary evidence suggests that the cross-link prevents nucleolytic cleavage of the oligonucleotide using snake venom phosphodiesterase.[117] A final advantage of this method is that the cross-linked helices are compatible with enzymatic radiolabeling techniques.

Over the past several years, several additional methods for incorporating disulfide cross-links into DNA and RNA have been developed. The sulfur-bearing nucleotides 6-thioinosine and 4-thiothymi-

dine were introduced at the penultimate positions of a 12 base pair duplex to form an intermolecular cross-link (**34**).[119] Although the desired cross-linked product was achieved in high yield, the reaction was sluggish (five days to completion) and the disulfide bonds were shown to be quite unstable to the small amounts of reducing agents present during standard end-labeling procedures. In a procedure that is reminiscent of the introduction of PEG and stilbene linkers, Gao and co-workers have bridged the 5′- and 3′-hydroxyls of helices with disulfide loops (**35**).[120,121] A disulfide cross-linked circular oligonucleotide has also been reported.[122] In this work, two C^5-(thiopropyne)thymidines (**36**) were incorporated within a single oligonucleotide strand and upon binding a complementary stand in the major groove a cross-link results. The disulfide-linked circle binds to its single-stranded host with an association constant of about 10^{17} M^{-1}: 3 orders of magnitude greater than the biotin:strepavidin couple. Lastly, Eckstein and co-workers have developed a method for incorporating disulfide cross-links into a hammerhead ribozyme.[123] Specifically, thiols were introduced at the 2′-hydroxyls of specific pyrimidine bases (**37**) to probe the active conformation of the ribozyme.



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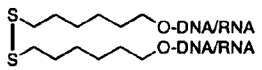

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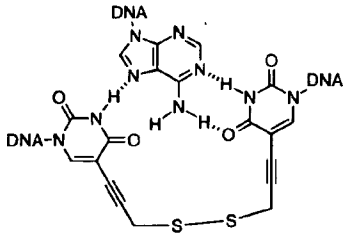

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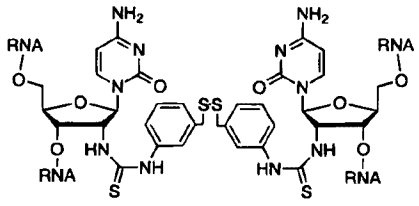

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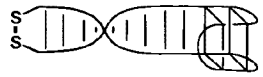
All of these methods are amenable to the stabilization of short aptamer sequences and structures. Nelson and co-workers have successfully introduced stilbene linkers at the termini of anti-Rev aptamers.[124] The cross-linked compounds bind Rev almost as well as the full-length *Rev responsive element* (*RRE*), which is over six times larger. In another example, Gao and co-workers linked the terminus of an anti-thrombin aptamer with either a PEG or disulfide cross-link (**35**) to give a the conformationally restrained G-tetramer (**38**).[74] Finally, Osborne and Ellington have developed a phosphoramidite (**39**) that can be used to introduce a disulfide cross-link at the termini of a helix during solid phase synthesis.[125] This reagent will be useful for constraining oligonucleotides that have difficulty forming properly and should pave the way for the synthesis of very short, very stable aptamers that are capped at both ends by disulfides.



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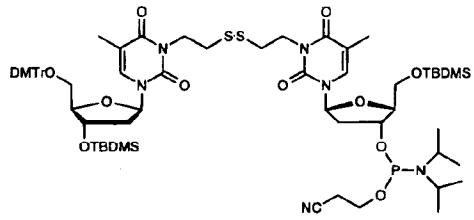

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VII. In Vivo Efficacy

In addition to blocking the activities of individual enzymes or regulatory proteins, aptamers can be used to inhibit particular aspects of cellular or viral metabolism in the same way that antisense oligonucleotides or triple helix agents are used to block genetic expression. For example, aptamers have been shown to block the function of a number of extracellular cytokines. When aptamers selected to bind to bFGF were assayed for their ability to inhibit bFGF binding to cell-surface receptors they were found to be effective at concentrations as low as 5 nM for low-affinity sites and 0.2 nM for high-affinity sites.[52] The anti-bFGF aptamers selected from modified RNA pools could inhibit interactions with receptors at concentrations as low as 1 nM for low-affinity sites and 3 nM for high-affinity sites.[78] An illustration of how anti-bFGF aptamers may function is given in Figure 9. Moreover, bFGF-induced cellular proliferation was blocked by aptamer concentrations of 50−100 nM. Similarly, anti-VegF aptamers selected from RNA pools inhibited receptor binding with an ED_{50} of 20−40 nM,[126] while the anti-VegF aptamers selected from modified RNA pools inhibited receptor binding at concentrations as low as 1 nM.[41] Finally, anti-IgE aptamers have been shown to block interaction with the Fce RI receptor and thereby inhibit IgE-mediated serotonin release from cells in tissue culture.[178]

Aptamer inhibition of enzymatic activity also has metabolic consequences. Aptamers selected against thrombin have been shown to block blood clotting in standard assays.[58] In addition, anti-thrombin aptamers can potentially prevent reocclusion of blood ves-

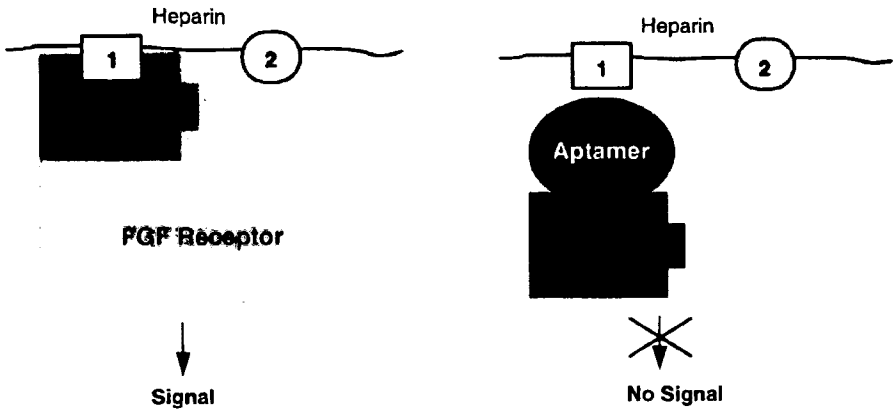


Figure 9. Illustration of aptamer inhibition of heparin binding sites. (Left) Receptor signaling requires formation of a trimeric complex where FGF-2 binds heparin at site 1 and to the FGF receptor (which binds the heparin at site 2). (Right) Aptamer binds to the heparin binding site of FGF-2 and prevents binding of the FGF receptor thereby suppressing the signaling event.

sels following coronary artery enzymatic thrombolysis or angioplasty.[127] In fact, in certain applications, such as hemodialysis or hemofiltration, the limited *in vivo* stability of the anti-thrombin aptamer may actually be advantageous, since clotting would be selectively inhibited for only a short period of time.[128] Anti-elastase aptamers that covalently link to their target were perfused into a rat lung and assayed for their ability to inhibit interleukin-1 (IL-1) induced, neutrophil-mediated damage (as monitored by edema-like weight gain).[72] The aptamer conjugated to the elastase inhibitor reduced the inflammatory response to near levels seen in the absence of IL-1 induction.

Finally, aptamers can modulate complex metabolic processes. Sequences selected from RNA and modified RNA populations to bind Rous sarcoma virus (RSV) can inhibit viral replication when mixed with the virus prior to infection.[81] An aptamer selected to bind a tRNA synthetase conjugated with its cognate amioadenylate was found to stimulate hydrolysis of noncognate aminoadenylates.[129] In other words, the aptamer enhanced the fidelity of error correction by an enzyme critical to protein biosynthesis. Anti-Rev aptamers have been shown to substitute for the Rev-binding element and efficiently facilitate RNA transport *in vivo*.[130]

In touting aptamers as potential pharmaceuticals we have obviously ignored a major problem: delivery. This problem has already been extensively considered (although not solved) in the development of antisense therapies, and many of the methods that have been developed for the delivery of antisense oligonucleotides are applicable to the delivery of aptamers. While assessments of delivery systems are outside the scope of this review, which focuses on selection principles and selected nucleic acids, we will present a brief rendition of the problems and prospects for aptamer delivery. It is unlikely that most nucleic acids will be orally available, although the possibility that some sequences or structures may directly enter the bloodstream should not be discounted. Therefore, aptamers against extracellular targets could be directly injected into an organism. Aptamers selected or engineered to resist degradation in sera can potentially be used "neat", without further protection. "Naked" or unprotected RNA or DNA aptamers may require further protection, most likely by conjugation to liposomes or other biopolymer carriers such as polyethylene glycol. Aptamers against intracellular targets can be delivered "endogenously" or "exogenously". Endogenous delivery is essentially gene therapy, with an anti-protein aptamer being transcribed in bulk from a strong polIII promoter. For example, anti-Rev aptamers expressed intracellularly have been shown to inhibit HIV-1 replication.[131] Exogenous delivery involves facilitated transport of aptamers across membranes. Polylysine has been successfully used to squelch the negative charges on nucleic acid backbones and enhance membrane transport.[132,133] A variety of cationic liposomal formulations have been developed for gene delivery, and these should be equally applicable to the delivery of aptamers.[134-136] Liposomes can even be targeted to specific cell types. For example, liposomes conjugated to folate can deliver nucleic acids to tumor cells.[137] Surprisingly, even unprotected nucleic acids may be delivered exogenously. Short oligonucleotides can be internalized by cellular pinocytosis[138] and endocytosis.[139] A variety of cell lines, including human cell lines such as HeLa, H9, and HL60, have been observed to take up exogenously introduced oligonucleotides rapidly and efficiently.[140-142] For example, antisense effects on intracellular targets can often be observed at external oligonucleotide concentrations ranging from 1−50 μM.[143] The internalized oligonucleotides typically localize to the nucleus.[144]

VIII. Conventional Drug Design

There are two ways in which *in vitro* selection can be used to further the development of conventional drugs (small organics). First, by helping to identify targets for drugs, either by defining nucleic acid targets or validating protein targets. Functional nucleic acids represent a largely untapped source of biological targets for drug development. Beyond their role as information-carrying macromolecules, there are numerous nucleic acids whose sequences and structures are crucial to viral, cellular, or organismal

function. Microorganisms have already discovered this fact and have evolved secondary metabolites, such as aminoglycoside antibiotics, that can bind to and inhibit cellular RNA molecules, such as ribosomal RNA. Aminoglycosides have also been shown to fortuitously inhibit other functional nucleic acids, including the Rev responsive element of HIV-1, the group I self-splicing intron, and the hammerhead ribozyme. *In vitro* selection can be used to define the binding domains of potential nucleic acid targets. In addition, the exquisite specificities of selected nucleic acids makes them useful tools for dissecting metabolism and validating whether other targets, such as proteins, are worthy candidates for drug development. Second, *in vitro* selection can be used to identify drugs themselves. Selection can assist in identifying putative anti-nucleic acid drugs, especially those derived from combinatorial chemical libraries.

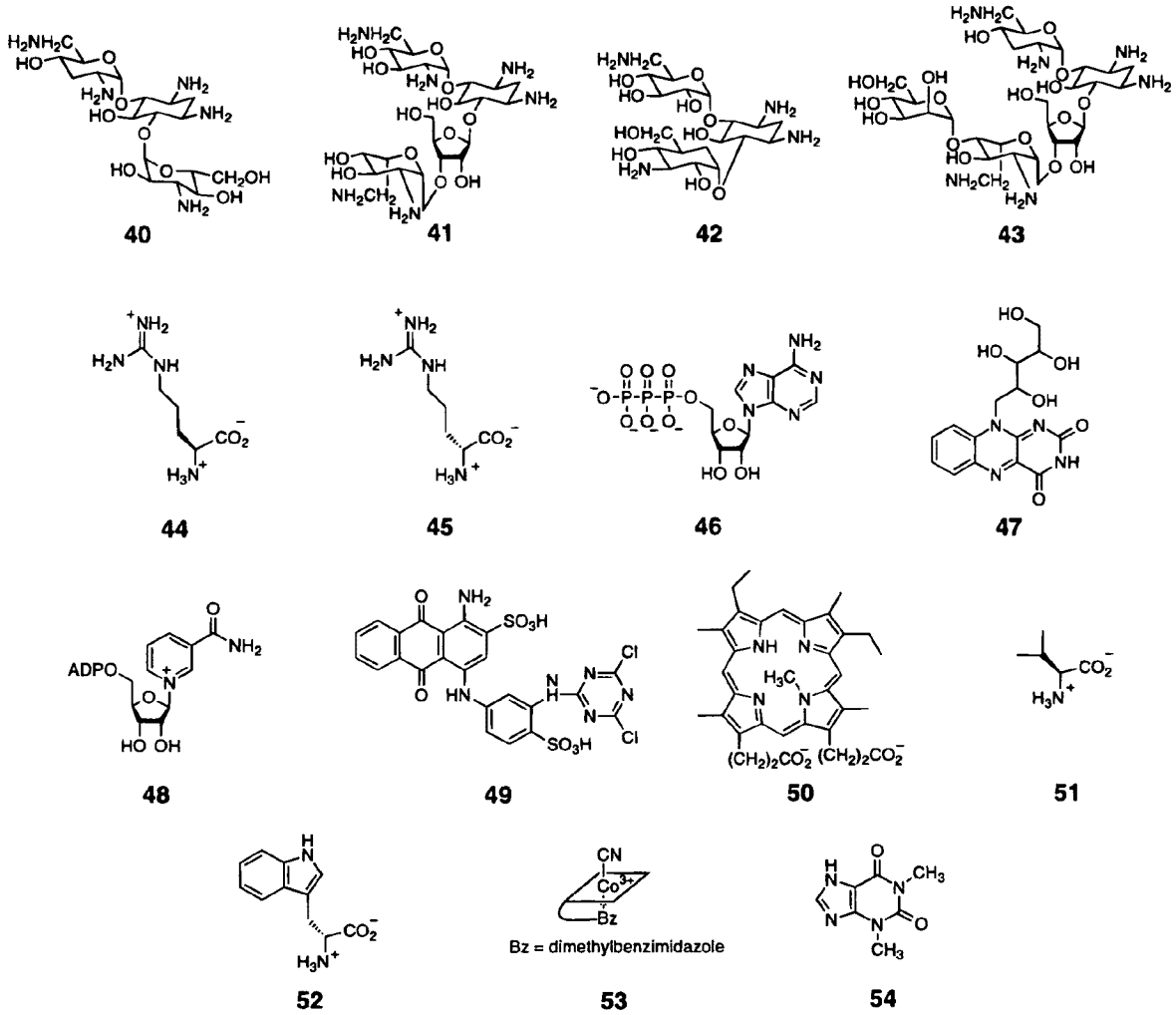
A. Target Identification

The sequences and structures of nucleic acid targets can be precisely defined by *in vitro* selection. As was the case with the anti-HIV aptamer described above, by sieving binding from nonbinding variants from a partially randomized population of sequences the relative importance of individual residues can be quickly established. Bartel and co-workers utilized this procedure to define the *Rev responsive element* of HIV-1.[145] The *RRE* had previously been partially defined by deletion analysis; a 67 nucleotide tract known to be important for function was synthesized so that individual positions contained 65% wild-type residues, 30% non-wild-type residues (10% of each non-wild-type residue), and 5% deletions. This population contained all single through hextuple variants of the 66 nucleotide sequence. Variants that could bind to Rev were selected by filtration. After three cycles of selection and amplification the population could bind better than the original wild-type *RRE*. The variants were sequenced, and 20 residues presented within a short stem-internal loop-stem structure were found to be important for Rev-binding activity. This delimited RNA is a potential target for drug development. In fact, the aminoglycoside antibiotic neomycin has been shown to fortuitously disrupt interactions between Rev and the *RRE*.[146] The neomycin binding site has been localized to the stem-internal loop-stem structure by modification interference analysis, and the drug was found to contact several of the residues revealed by selection experiments to be important for function. Baskerville and co-workers have carried out similar studies with the Rex-binding element of HTLV-I and have determined that residues within a stem containing two base bulge loops are important for Rex-binding activity.[147] Completely random sequence pools can also be used to define functional RNA sequences, providing that the random sequence tracts are short. For example, two hexanucleotide loops in the hepatitis B virus encapsidation signal were separately randomized, and functional variants were selected *in vivo* based on their ability to be encapsidated.[148] Sequence comparison of selected clones revealed that most of the randomized positions had reverted to wild-type and hence were essential for RNA packaging. These results suggest that the virus might not readily mutate to avoid inhibition by a drug that targeted one of the loops. However, it should be noted that while selection strategies can provide insights into the sequences and structures of natural RNAs, they do not always return wild-type binding sites. When the neomycin binding site of ribosomal RNA was partially randomized and selected for its ability to interact with neomycin, the sequences that were recovered bore no resemblance to the wild-type.[149] Instead, a motif previously observed in anti-neomycin aptamers selected from completely random sequence pools was recovered.[150]

Selection can also be used to define DNA targets. Because DNA binding sites for proteins are located within a similar structural context (the double helix), *in vitro* selection experiments typically start with a completely random pool of double-stranded DNA. Binding sites for several members of the basic helix-loop-helix family of transcription factors, including MyoD, have been defined by this method.[25] Interestingly, it looks as though the recognition sequences for homo- and heterodimeric transcription factors may be modular, with each protein monomer recognizing its particular half-site. These results have recently been extended to several other members of the bHLH family, including the Ah receptor, the Ah receptor nuclear transport protein, and single-minded protein.[151] These results are especially significant given the recent development of "lexitropsins" (sequence-reading compounds) that can specifically recognize stretches of DNA that are similar in length to the bHLH family binding sites.[152,153]

Selection can help to define targets other than nucleic acids. As we have seen, aptamers can frequently distinguish between closely related protein targets. It may therefore be possible to use selected nucleic acid shapes as probes that assess whether or not drugs that bind a particular site are likely to be cross-reactive. *In vitro* selection experiments may even be able to uncover binding specificities that were previously unknown. Experiments with polyprimidine tract-binding proteins U2AF,[65] Sex-lethal, and polyprimidine tract-binding protein (PTB) provide a particularly good example of how selections can reveal new specificities.[154] All of these proteins bind to predominantly uridine-rich sequences. Uridine-rich sequences selected by $U2AF^{65}$ were similar to polyprimidine tracts found in a variety of genes, consistent with the role of $U2AF^{65}$ as a general splicing factor. In contrast, Sex-lethal yielded a specific uridine-rich sequence similar to the pyrimidine tract of substrates such as pre-mRNA. Selection against PTB yielded a distinct consensus sequence found within the introns of rat α- and β-tropomyosin. Similarly, selections that targeted the ASF/SF2 (alternative splicing factor/splicing factor 2) and SC35 (splicing component 35) splicing factors resulted in the isolation of distinct consensus-sequence motifs, indicating that these factors may not be functionally redundant.[155] Aptamers can not only probe the specificities of targets related by history or function, but can also be used to probe different states or conformations of the same target. For example,

Chart 2. Structure of Small Molecule Ligands Used as Aptamer "Guests"



selections that targeted whole ribosomes identified different aptamers depending on whether ribosomal protein S1 was present or absent from the complex.[156]

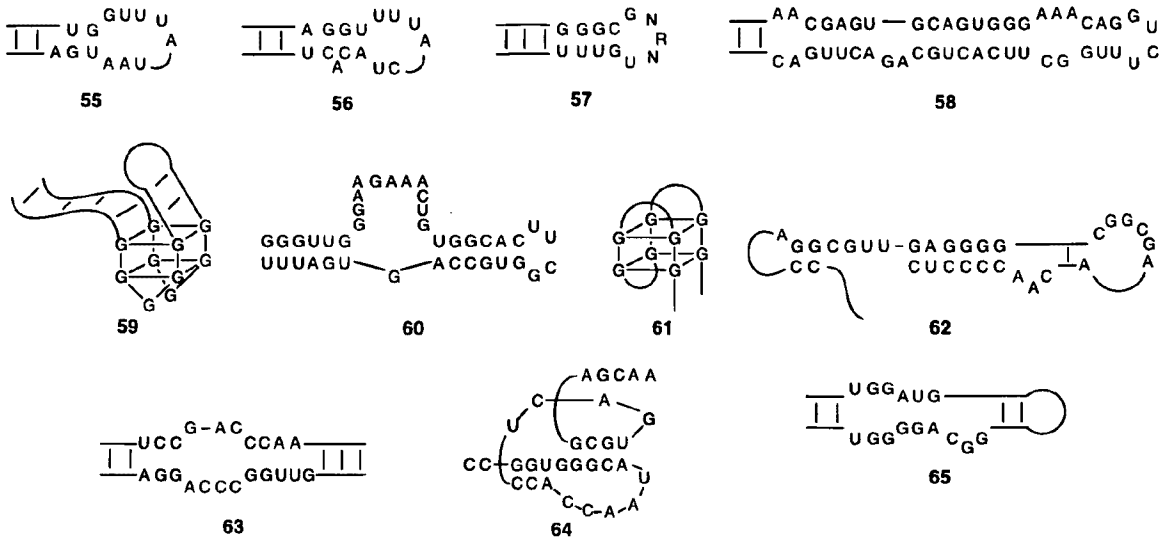
While splicing factors and the ribosome were already known to bind nucleic acids, aptamers could similarly be used to probe the specificities of non-nucleic acid binding protein targets. For example, while many cytokines and receptors possess what are nominally known as heparin binding sites, the architectures of these sites and their natural oligosaccharide ligands may be quite different. Anti-FGF aptamers compete with heparin for binding to FGF,[52,78] anti-VegF aptamers compete with heparin for binding to VegF,[126] and anti-thrombin aptamers selected from RNA pools compete with heparin for binding to thrombin,[157] but these nucleic acid ligands are not known to cross-recognize their protein targets. The structural and functional differences between these aptamers may therefore signal differences in oligosaccharide binding site specificities. To the extent that aptamers have *in vivo* efficacy, such binding differences can potentially be translated into differential activities in biological assays. In this way, aptamers can be used to validate a protein target by determining whether inhibition of a given protein will produce a given physiological effect. In effect, even when aptamers may not be suitable drugs in and of themselves, they can be used as drug mimics.

B. Drug Design

In vitro selection can also be used to discover drugs rather than define targets. The discovery process can be applied either to existing drugs or to novel compounds that bind nucleic acids. Nucleic acid sequences that are discovered to bind to existing drugs may have counterparts in organismal or viral genomes, and hence may reveal new targets for compounds with proven bioavailabilities and pharmacokinetic properties.

While we have so far focused primarily on large biomolecular targets, aptamers that specifically bind to small molecular "guests" can also be selected (Chart 2). For example, the aminoglycoside antibiotics tobramycin (**40**), neomycin (**41**), kanamycin (**42**), and lividomycin (**43**) have elicited tight and specific aptamers. Wang and Rando examined the binding interactions of tobramycin with an RNA pool con-

Chart 3. Consensus Sequences and Secondary Structures of Aptamers Selected To Bind Small Molecule Ligands



taining a 60-base random site.[158] In their study they found a number of aptamers that weakly bound (~10 mM) tobramycin with no apparent consensus sequence. However, upon increasing the stringency of selection by lowering the amount of ligand attached to a solid support, the affinity for binding increased with each cycle until the binding was in the submicromolar range. Cloning of the aptamers revealed two similar stem-loop consensus sequences (**55** and **56**) (Chart 3). Similarly, selections that targeted neomycin generated a consensus motif (**57**) that could interact tightly with the antibiotic ($K_D \approx 100$ nM) and discriminate against the aminoglycoside paromomycin (**66**), which differs by only a single amino moiety, by over 100-fold.[150] Finally, aptamers that can form complexes with kanamycin (**42**) and lividomycin (**43**) have been selected from a random sequence RNA pool.[159] After several cycles, the selected species had affinities (aggregate $K_D \approx 300$ nM) and specificities for the aminoglycosides that rivaled those of ribosomal RNA. As was the case for the early cycles of the selections that targeted tobramycin, there were numerous ($\approx 10^6$ estimated) aptamers in the selected population and no clear consensus sequence. The fact that multiple, different sequences exist that have the same binding characteristics as a known drug target (ribosomal RNA) implies that there may be many cellular or viral RNA molecules that could also be bound and affected by aminoglycosides. A map of aminoglycoside structure to RNA sequence might provide new targets for old drugs. In order to test this "reverse drug discovery" method, the lividomycin aptamers were further selected until a consensus sequence emerged (**58**).[160] The consensus sequence was compared with all sequences in Genbank, and a number of potential targets, including RNAs from *Haemophilus influenzae* and the parasite *Leishmania*, were identified. Because small molecules recognize relatively small motifs (10−30 bases), there is a good chance that at least one gene or structural motif will be discovered.

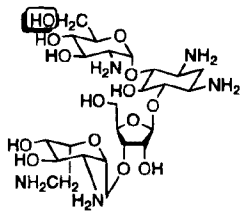
There is reason to believe that there may be a large number of drug leads beyond aminoglycosides that might be candidates for such reverse drug discovery methods. Some of the earliest work in understanding the applications of selection techniques was performed with small organic dyes (**49**) which previously had not been known to interact with nucleic acids.[29,161] Natural as well as synthetic compounds have also been shown to have surprising affinities for particular RNA molecules. Lauhon and Szostak targeted the biological cofactor, nicotinamide adenine dinucleotide (NAD) (**48**).[162] Selection of an RNA pool containing an 80-base random region against NAD using nicotinamide mononucleotide (NMN) (**67**) as an eluant resulted in nearly 50% of the population being selectively bound after eight cycles of selection. One of the clones isolated from the final cycle could discriminate between the oxidized cofactor, NAD^+ (K_D of 2.5 mM), and the reduced cofactor, NADH (K_D of 37 mM), which differ by only a single hydride. The selectivity exhibited by this RNA is similar to that seen for protein dehydrogenases and reductases. Two further examples of aptamers that bind small organic molecules with particularly high affinities and specificities were afforded by selections that targeted ATP (**46**) and the drug theophylline (**54**). The anti-ATP aptamer (**59**) selected by Sassanfar and Szostak has a K_D for ATP of 0.7 μM and discriminates against a closely related substrate, dATP, by a factor of over 1000.[163] The anti-theophylline aptamer (**64**) selected by Jenison and co-workers has a K_D for theophylline of 320 nM.[164] During the selection procedure, aptamers that could also recognize caffeine (**68**), which differs from theophylline by the addition of a single methyl group, were removed from the population. As a result of this counterselection, the antitheophylline aptamer can discriminate against caffeine by a factor of over 10 000, a value better than that observed for comparable monoclonal antibodies. A variety of other small molecule targets, from compounds as small as zinc[165,166] to compounds as large as porphyrins (**50**)[167]

Table 5. Aptamers Selected To Bind Small Molecule Ligands

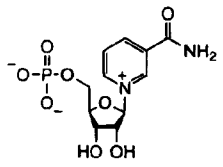
ligand	entry number[a]	pool	selected aptamer[b]	conjugate ligand K_D (μM)	specificity (nonconjugate)	ref
tobramycin	**40**	RNA	**55 and 56**	0.006	900 (6′-*N*-4-pyreneacetyl tobramycin)	158
neomycin	**41**	RNA	**57**	0.10	100 (paromomycin)	150
kanamycin	**42**	RNA	N/A[c]	≈0.3	10−100 (kanamycin B)	159
lividomycin	**43**	RNA	**58**	≈0.3	≈1 (paromomycin)	159
L-arginine	**44**	RNA	**6**	10.0	120 (citrulline)	50
D-arginine	**45**	RNA	**3**	200	1.7 (L-arginine)	47
ATP	**46**	DNA	**59**	6	N/A	82
	46	RNA	**60**	0.7	1430 (dATP)	163
riboflavin	**47**	RNA	**61**	1−5	~1 (5-deazariboflavine)	162
nicotinamide (NAD)	**48**	RNA	**62**	2.5	14.8 (NADH)	162
reactive blue 4	**49**	RNA	**63**	600	85 (cibacron blue)	29
N-methylmesoporphyrin IX	**50**	DNA	N/A	0.5	3 (mesoporphyrin IX)	167
L-valine	**51**	RNA	N/A	2900	62 (D-valine or leucine)	*d*
D-tryptophan	**52**	RNA	N/A	18.0	670 (L-tryptophan)	*e*
vitamin B12 (cyanocobalamin)	**53**	RNA	**64**	0.088	11400 (adenosyl-cobalamin)	168
theophylline	**54**	RNA	**65**	0.32	10900 (caffeine)	164

[a] See Chart 2 for ligand structures. [b] See Chart 3 for aptamer structures. [c] Not available or determined. [d] Majerfeld, I.; Yarus, M. *Nature Struct. Biol.* **1994**, *1*, 282. [e] Famulok, M.; Szostak, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 3990.

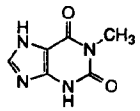
and vitamin B12 (cyanocobalamin, **53**),[168] have successfully elicited aptamers. Selections that targeted small molecules was recently reviewed by Ellington and Table 5 provides an updated summary of results.[9]



66

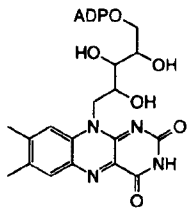


67



68

Not only are the binding characteristics of selected nucleic acids consonant with the use of small molecules as anti-nucleic acid drugs, but the recently determined structures of aptamer:ligand complexes have features that are reminiscent of protein:drug complexes. Burgstaller and Famulok selected aptamers that could form complexes with the cofactor FMN (**69**), and used comparative sequence analysis to derive a minimal, functional motif (**70**).[169] Fan and co-workers have determined the NMR structure of the complex.[170] Recognition is based on stacking interactions below and above the aromatic, planar cofactor; for example, a base triple (A:U:G) lies parallel and adjacent to the bound FMN. Some specificity for FMN is provided by two hydrogen bonds between the cofactor and the Hoogsteen face of an adenosine that lies in the same plane. These features are similar to those observed in complexes between trimethoprim and dihydrofolate reductase. Famulok also selected aptamers that could bind to either citrulline (**71**) or arginine (**44**), depending on the identity of three positions in the selected motif.[50]



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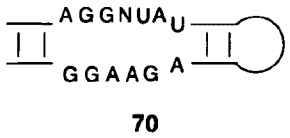
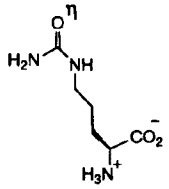


70

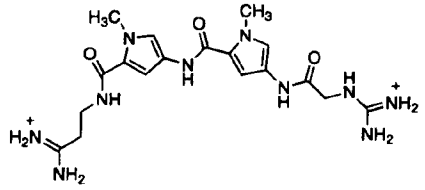


71

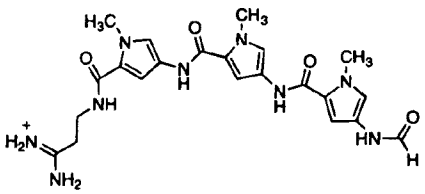
Modification interference experiments carried out with a minimal binding motif revealed that an internal loop structure was critical for interactions with the amino acids, and that the conformation of the aptamer changed on complexation.[171] The NMR structures of the anti-citrulline and anti-arginine aptamers complexed with their respective ligands

have been determined.[172] Five guanosine residues come together to form the base of a binding pocket; the aliphatic side chains of the amino acids stacks over this base. The binding pockets are lined with hydrogen-bond donors and acceptors that contact the side chains of the amino acids. In the anti-citrulline aptamer, O^{η} is contacted by the hydrogen at the N^{δ} position of U13 and the exocyclic amine of G29; in the anti-arginine aptamer, the corresponding N^{η} position is contacted instead by the N^{δ} of C13: a hydrogen-bond donor has been exchanged for a hydrogen-bond acceptor. Surprisingly, there are no apparently electrostatic contacts between the aptamer and arginine, and the interactions are unlike those seen between nucleic acids and the arginines or nucleic acid binding proteins. The structure of the anti-ATP aptamer has also been solved by NMR.[173] As was the case with the anti-amino acid aptamers, the ligand is bound in a loop between two stems. However, the overall architecture of the complexes are quite different. The adenine base is intercalated between two purines, A10 and G11; G11 pinches off a short loop structure by forming a non-Watson–Crick interaction with G7. This loop bears a striking resemblance to a well-known and previously characterized RNA structure, the GNRA tetraloop, with the adenosine ligand base-pairing with G8 and acting as the 3′ most adenosine of the loop. The right-hand stem of the structure is also capped by a non-Watson–Crick interaction (between G17 and G34), and provides additional hydrogen bonds to the N^{δ} position of adenine and to the free hydroxyls of ribose. The pocket that is formed contacts nearly half of the available surface area of the ligand, and satisfies almost every possible hydrogen bonding partner.

The selectivities and structures of aptamers that bind small molecules lend credence to the suggestion that *in vitro* selection can help to form the basis of a drug development program.[174] While there are numerous compounds that have been designed to bind nucleic acids, their known affinities and specificities are too low to ensure their success as drugs. For example, netropsin (**72**) and distamycin (**73**) have



72



73

served as starting points for attempts for the design and construction of "lexitropsins", but these compounds and their derivatives generally have dissociation constants from nucleic acid targets (A:T tracts) in the micromolar range[175] and discriminate against noncognate sequences by factors of only 10–100.[176] The aminoglycosides in general have slightly higher affinities for their targets, but are still far too catholic in their preferences for nucleic acids. However, recent attempts to augment the number of residues recognized by the minor-groove binding lexitropsins and to use aminoglycosides as synthetic scaffolds for the production of novel pharmacophores are promising. These efforts can be facilitated by an examination of the problem from the point of view of the biopolymer "host," rather than the organic "guest". *In vitro* selection can define and rank the sequence motifs that are recognized by anti-nucleic acid drug leads, such as lexitropsins or aminoglycosides. As new synthetic derivatives are produced, their specificities can be determined, and the process iterated to hone interactions between a drug and its desired target. A database that maps compound structure to nucleic acid sequence should eventually prove invaluable in the *de novo* design of specific anti-nucleic acid compounds.

The fact that a plethora of compounds have successfully elicited aptamers immediately suggests that many excellent anti-nucleic acid drug leads are currently unknown. *In vitro* selection could therefore be routinely used to screen existing chemical libraries, providing information as to which compounds bind which nucleic acid sequences or structures. Given the defined nature of the selection process, it is likely that the entire procedure, including sequence acquisition, could be automated. More importantly, nucleic acid selections could be carried out against mixtures of compounds. This should allow whole-scale screens of chemical libraries by random sequence nucleic acid pools: the compounds with the highest affinity for nucleic acids in general would elicit binding species; these binding species would compete with one another until only the best pairings between potential anti-nucleic acid drugs and aptamers remained. Further definition of the selected sequences by chemical probes or structural studies may then suggest rational chemical modifications that would increase the affinities of anti-nucleic acid drug leads. Screens of combinatorial chemistry libraries could be combined with the "reverse drug discovery" method to yield fortuitous "hits" in cellular or viral genomes.

C. Mimetics

Finally, aptamers themselves may serve as scaffolds for the design and synthesis of small organics. Just as peptidomimetics have been synthesized on the basis of peptides selected from phage display libraries or protein structures, so too can nucleic acid mimetics be synthesized on the basis of the sequences and structures of aptamers. Understanding how functional groups on the bases, sugars, and phosphates of aptamers are positioned in a protein active site will obviously be aided by a determination of either the aptamer structure or, better, the structure of the complex. If these structures are known, then the functional groups can be introduced in a similar

configuration on a small organic backbone. Unfortunately, as is the case with proteins, deriving the structures of nucleic acids and nucleic acid complexes by NMR or crystallography is a somewhat laborious task. However, unlike with proteins, it may be possible to readily model the structures of nucleic acids. While the overall form of a protein is largely a consequence of tertiary structural interactions, the overall form of a nucleic acid is largely a consequence of secondary structural interactions. Nucleic acid secondary structures are in turn primarily dependent on Watson–Crick and other base pairings that have relatively defined structures. Thus, once the secondary structure of a nucleic acid is known, and if some tertiary structural contacts are known or suspected, then a three-dimensional structural model can be readily generated. An example of this type of analysis has already been carried out with an aptamer that binds HIV-1 Rev.[177]

A more intriguing prospect for drug design involves using nucleic acids as scaffolds for the presentation of limited chemical libraries. The fact that selections can be carried out with modified nucleic acids immediately suggests that the modifications could be geared toward applications other than stability. For example, in order to "fill" a hydrophobic pocket on a protein, aptamers could be selected from libraries derivatized with hydrophobic functional groups. The selected sequences could then be used to model or physically map how the hydrophobic functional groups were positioned, and mimetics designed. In this case, though, the mimetics would not rely as heavily on the nucleic acid backbone, but rather would attempt to string together the identities, positions, and orientations of the hydrophobic functional groups. In this way, nucleic acid libraries might effectively become replicating chemical libraries.

IX. Conclusion

In vitro selection experiments can provide both lead compounds for drug development and insights into methods for drug discovery. Aptamers generally bind to their targets with high affinities and specificities, and can be converted into smaller, more stable compounds by a variety of chemical modifications. *In vitro* selection can be thought of as an adjunct to combinatorial chemistry. Traversing functional "landscapes" with random sequence nucleic acids is roughly analogous to traversing the same "landscapes" with combinatorial chemical libraries, and some of the same principles may generally apply. Selection can be used to define novel nucleic acid targets for drug discovery or design, and to validate more popular protein targets. The interplay between combinatorial chemical libraries and selected nucleic acids may yield "codes" for recognition that will serve as the basis for rational drug design.

X. Acknowledgments

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EXHIBIT C

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Structure-Switching Signaling Aptamers

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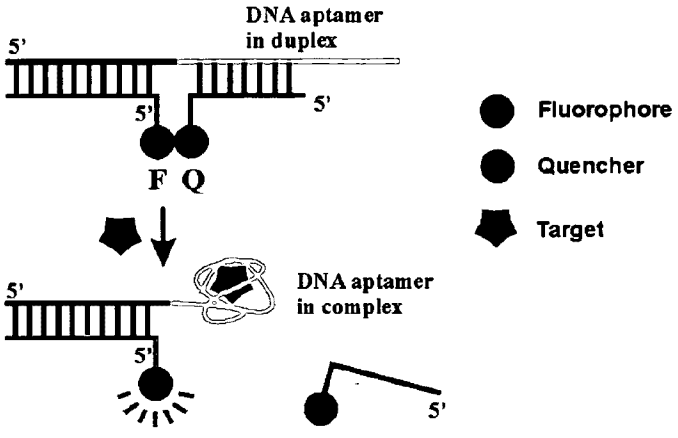

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Structure-Switching Signaling Aptamers

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Abstract: Aptamers are single-stranded nucleic acids with defined tertiary structures for selective binding to target molecules. Aptamers are also able to bind a complementary DNA sequence to form a duplex structure. In this report, we describe a strategy for designing aptamer-based fluorescent reporters that function by switching structures from DNA/DNA duplex to DNA/target complex. The duplex is formed between a fluorophore-labeled DNA aptamer and a small oligonucleotide modified with a quenching moiety (denoted QDNA). When the target is absent, the aptamer binds to QDNA, bringing the fluorophore and the quencher into close proximity for maximum fluorescence quenching. When the target is introduced, the aptamer prefers to form the aptamer-target complex. The switch of the binding partners for the aptamer occurs in conjunction with the generation of a strong fluorescence signal owing to the dissociation of QDNA. Herein, we report on the preparation of several structure-switching reporters from two existing DNA aptamers. Our design strategy is easy to generalize for any aptamer without prior knowledge of its secondary or tertiary structure, and should be suited for the development of aptamer-based reporters for real-time sensing applications.

Introduction

Aptamers are single-stranded nucleic acids isolated from random-sequence nucleic acid libraries by "in vitro selection".[1,2] To date, numerous aptamers have been created for a broad range of targets, including metal ions, small organic compounds, metabolites, and proteins.[3,4] The tight-binding capabilities of both DNA and RNA aptamers have been demonstrated in numerous cases including a 2′-aminopyrimidine-containing RNA aptamer for vascular permeability factor/vascular endothelial growth factor with a K_d of 0.14 nM,[5] a 2′-fluoro-modified RNA aptamer for the human keratinocyte growth factor with K_d of 0.3 pM,[6] and a DNA aptamer for platelet-derived growth factor-AB with subnanomolar affinity.[7] Aptamers can also be made to possess a high binding specificity, exemplified by an anti-theophyllin RNA aptamer[8] that displays a >10 000-fold discrimination against caffeine (which differs theophyllin by a methyl group) and an anti-L-arginine RNA aptamer that exhibits a 12 000-fold affinity reduction toward D-arginine.[9] The target versatility, the high binding affinity and specificity, along with the simplicity of in vitro selection, make aptamers attractive as molecular tools for bioanalytical applications. In such cases, it is advantageous if aptamers are able to report on target presence by real-time fluorescence signaling without a need for complex separation steps.

Standard DNA and RNA molecules do not contain intrinsically fluorescent groups. To make aptamers fluoresce, it is necessary to modify aptamers with extrinsic fluorophores. Considerable research activities aiming at designing real-time signaling aptamers have been reported recently. One strategy is to covalently attach a fluorophore at a location of an aptamer that will undergo a target-induced conformational change.[10,11] Such reporters can be created either by rational design if tertiary structure information is available[10] or by in vitro selection using a fluorophore-labeled library.[11] A critical assumption in this approach is that the conformational change might substantially alter the electronic environment of the attached fluorophore to cause a significant change in its fluorescence property. Because of the difficulty in precisely predicting (1) whether the attachment site will undergo a significant conformational change upon target binding and (2) whether such a change could indeed alter the fluorescence property of the attached fluorophore, many

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rationally designed constructs[10] or selected aptamers[11] may have to be tested before a desirable signaling aptamer can be obtained. Therefore, this strategy is not easy to generalize. Furthermore, the known signaling aptamers made by this approach usually exhibit fairly small fluorescence enhancements upon target binding (typically below 2-fold at saturating target concentrations[10,11]) and consequently, their detection sensitivity is relatively low.

Other studies have focused on designing molecular beacon-based signaling aptamers (denoted "aptamer beacons")[12-16] through the adaptation of the molecular beacon concept originally designed for the detection of nucleic acid targets by nucleic acid hybridization.[17] Yamamoto et al. reported the first aptamer beacon designed from an RNA aptamer that interacts with the Tat protein of HIV.[13] These researchers split the aptamer into two molecules, one of which was formulated into a hairpin-shaped beacon molecule (after the addition of a few nucleotides to tie the two ends of the RNA into a hairpin structure and the attachment of a fluorophore at one end of the RNA and a quencher at the other end). In the absence of Tat, the two RNA molecules exist independently; the beacon half of the aptamer adopts the hairpin structure, emitting a low level of fluorescence. When Tat is introduced, the beacon changes its structure in order to engage the other half of the aptamer for binding to Tat; the disruption of the hairpin structure causes physical separation of the fluorophore-quencher pair, resulting in a fluorescence enhancement. The successful design of the above signaling aptamer is achieved because the original RNA aptamer has a unique secondary structure that contains a long stretch of paired nucleotides to permit the splitting of the aptamer into two molecules. Therefore, it can be difficult to use the same strategy for other aptamers that lack such a secondary structure feature. An alternative molecular beacon strategy has been reported by Hamaguchi et al.[12] in which they place an intact aptamer as the loop segment of a molecular beacon. However, this strategy is difficult to generalize as well, particularly for large aptamers and the aptamers in which the two ends of the aptamer sequence do not move away from each other after target binding (e.g., the anti-ATP DNA aptamer[18,19]). Moreover, tying the two ends of an aptamer into a hairpin structure could significantly alter the correct tertiary folding of the aptamer and consequently, such a modified aptamer may lose its binding ability. For example, only one of the three anti-thrombin aptamer beacons designed by Hamaguchi et al.[12] based on a known anti-thrombin DNA aptamer[20] was able to retain the thrombin-binding ability, whereas the other two failed to interact with thrombin completely.[12] Considering that aptamers have variable sizes and different kinds of secondary structures and that many aptamers may not have an easily determined secondary structure, there is an obvious need to establish a signaling-aptamer designing strategy that is easy to generalize and has little restrictions on the size and secondary structure of aptamers.

Herein, we describe a simple and general approach for preparing solution-based signaling aptamers that function by a coupled structure-switching/fluorescence-dequenching mechanism. Our strategy exploits the unique ability of each DNA aptamer to adopt two distinct structures—a DNA duplex with a complementary DNA sequence, and a tertiary complex with the target for which the aptamer is created. Our signaling aptamers take advantage of target-induced switching between a DNA/DNA duplex and a DNA/target complex. Generation of a signal upon formation of the DNA/target complex is obtained by using a fluorophore-labeled DNA aptamer and a small complementary oligonucleotide that is covalently modified with a quencher (denoted QDNA). In the absence of the target, the aptamer naturally binds to the QDNA, bringing the fluorophore and the quencher into close proximity for highly efficient fluorescence quenching. When the target is introduced, the aptamer prefers to form the aptamer-target complex rather than the aptamer-QDNA duplex, triggering the release of the QDNA from the fluorophore-labeled aptamer. The dissociation of the QDNA is accompanied by the increase of fluorescence intensity because of fluorescence dequenching. On the basis of this strategy, we have successfully engineered several fluorescent reporters from two existing DNA aptamers, one that is specific for ATP and the other that binds thrombin.

Experimental Section

DNA Oligonucleotides and Chemical Reagents. Standard and modified DNA oligonucleotides were all prepared by automated DNA synthesis using cyanoethylphosphoramidite chemistry (Keck Biotechnology Resource Laboratory, Yale University; Central Facility, McMaster University). 5′-Fluorescein and 3′-DABCYL (4-(4-dimethylaminophenylazo)benzoic acid) moieties were introduced using 5′-fluorescein phosphoramidite and 3′-DABCYL-derivatized controlled pore glass (CPG) (Glen Research, Sterling, Virginia) and were purified by reverse phase HPLC. HPLC separation was performed on a Beckman-Coulter HPLC System Gold with a 168 Diode Array detector. The HPLC column was an Agilent Zorbax ODS C18 Column, with dimensions of 4.5 × 250 mm and a 5 μm bead diameter. A two-solvent system was used for the purification of all DNA species, with solvent A being 0.1 M triethylammonium acetate (TEAA, pH 6.5) and solvent B being 100% acetonitrile. The best separation results were achieved by a nonlinear elution gradient (10% B for 10 min, 10%B to 40%B over 65 min) at a flow rate of 0.5 mL/min. The main peak was found to have very strong absorption at both 260 and 491 nm. The DNA within 2/3 of the peak-width was collected and dried under vacuum. Unmodified DNA oligonucleotides were purified by 10% preparative denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE), followed by elution and ethanol precipitation. Purified oligonucleotides were dissolved in water and their concentrations were determined spectroscopically. Human factor Xα and human factor IXα were purchased from Haematologic Technologies (Essex Jct., VT). Human thrombin, bovine serum albumin (BSA), adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), deoxyadenosine 5′-triphosphate (dATP), uridine 5′-triphosphate (UTP), guanosine 5′-triphosphate (GTP), cytosine 5′-triphosphate (CTP),

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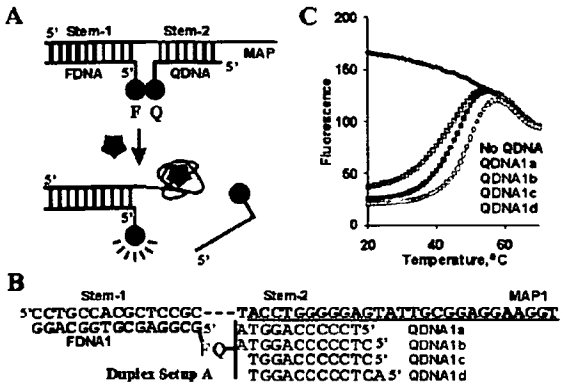


Figure 1. Design of structure-switching signaling aptamers. (A) Working principle. A tripartite, fluorescence-quenching, two-stem duplex assembly can be constructed using a fluorophore (F)-containing FDNA (in green), a quencher (Q)-containing QDNA (in red) and an unmodified DNA molecule (MAP) that contains an FDNA-binding sequence (in black) and a target-binding motif (in light blue). When the target (blue star) is introduced, the duplex structure is transformed into the target-aptamer complex with a concomitant release of QDNA and an enhancement of fluorescence intensity. (B) An ATP-binding aptamer as a model system. The original 27-nt aptamer (underlined) is appended with a sequence at the 5′-end for the formation of the 15-bp stem-1 with FDNA1. Four QDNAs, each containing a DABCYL at the 3′-end, were used to establish a suitable stem-2. (C) Thermal denaturation profiles of DNA solutions containing FDNA1 and MAP1 (filled black diamonds) as well as FDNA1 and MAP1 with one of the following QDNAs: QDNA1a (red open squares), QDNA1b (filled green triangles), QDNA1c (filled blue squares), and QDNA1d (open purple circles). The experiments were carried out as described in the experimental protocol section.

were purchased from Sigma and their solution concentrations were determined by standard spectroscopic methods.

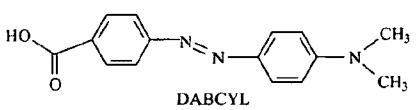
Fluorescence Measurements. The following concentrations of oligonucleotides were used for fluorescence measurements (if not otherwise specified): 40 nM for FDNA1, 80 nM for the aptamers (MAPs) and 120 nM for the quenchers (QDNAs). The ratio of FDNA: MAP:QDNA was set to be 1:2:3 to ensure a low background signal. Under this setting, the vast majority of FDNA molecules would form a duplex structure with MAP and the resulting FDNA-MAP duplexes would also be able to engage a QDNA molecule for fluorescence quenching. DNA solutions also contained 300 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tris·HCl (pH 8.3) for the ATP reporters, and 5 mM KCl, 1 mM $MgCl_2$ and 20 mM Tris·HCl (pH 8.3) for the thrombin reporter. The fluorescence intensities were recorded on a Cary Eclipse Fluorescence Spectrophotometer (Varian) with excitation at 490 nm and emission at 520 nm. To obtain the thermal denaturation profile of a particular reaction mixture, the DNA solution was heated to 90 °C for 5 min, and the temperature was then decreased from 90 °C to 20 °C at a rate of 1 °C/min. The fluorescence intensity was recorded automatically for every 1 °C drop in temperature. Measurements of fluorescence intensities from specific samples are detailed in each figure legend.

Results

Signaling Aptamer for ATP Sensing. A scheme demonstrating the concept of the duplex-to-complex structural transition coupled with fluorescence dequenching is shown in Figure 1A. Three synthetic DNA oligonucleotides are used: the first one is modified with a fluorophore at the 5′-end (denoted FDNA), the second one is labeled with a quencher at the 3′-end (denoted QDNA), and the third DNA molecule (denoted MAP) consists of an aptamer domain and an FDNA-binding motif. The QDNA is complementary to the aptamer in the sequence segment near the FDNA-binding motif. In the absence of the target, the three DNA molecules are expected to assemble into the tripartite duplex structure in which the fluorophore and the quencher are situated in close proximity, leading to efficient fluorescence quenching. Because the aptamer domain has a propensity to form the aptamer-target complex, the introduction of the target into the DNA mixture should cause the aptamer to release the QDNA in favor of the target, producing a large increase of fluorescence intensity.

Figure 1B illustrates an ATP-binding DNA aptamer as a test case. The original DNA aptamer was created by Huizenga and Szostak through in vitro selection[18] and it was found from an NMR study that the aptamer forms a tertiary complex with two ATP molecules.[19] We added an arbitrarily chosen 15-nt GC-rich sequence onto the 5′-end of the aptamer for FDNA1 binding. FDNA1 (modified with 5′-fluorescein) and its complementary sequence were found to form a DNA duplex with a measured melting point of 68 °C (in 0.5M NaCl, data not shown). Therefore, stem-1, which is formed between FDNA1 and MAP1, is sufficiently stable in the temperature range used for aptamer binding. A single nucleotide, T16 of MAP1, was introduced to separate the FDNA1 binding domain and the aptamer domain to minimize the potential steric interference between the two domains in the folded tertiary structure.

Several 3′-DABCYL-modified oligonucleotides (QDNA1a to QDNA1d) were tested as quenchers [DABCYL: (4-(4-dimethylaminophenylazo)benzoic acid; structure shown below), and their ability to form stem-2 with MAP1 was judged by the thermal denaturation profiles shown in Figure 1C. QDNA1b (filled green triangles) and QDNA1d (open purple circles) were the two most effective quenchers in the group and had apparently equal quenching efficiency. This observation is not a surprise considering that the 12-bp stem-2 formed by both QDNAs has the same base composition. The two 11-nt QDNAs, however, exhibited different quenching efficiencies with QDNA1c being more effective than QDNA1a. This is likely due to the increased GC content of QDNA1c as it contains 8 GCs (7 GCs in QDNA1a). We chose to use QDNA1c to test the ATP induced structure switching because QDNA1c forms a stem-2 that is almost as stable as those formed by the two 12-nt QDNAs at low temperatures (20−30 °C). However, because the QDNA1c has a less stable stem-2 (whose melting point is ~3 °C lower than those by the two 12-nt QDNAs), it should dissociate more easily from MAP1 in the presence of ATP and thus be a more sensitive reporter.



DABCYL

Evidence for Structure Switching. FDNA1-QDNA1c-MAP1 tripartite system is denoted as ATP Reporter A (Figure 2A). We used a series of temperature-changing fluorescence assays to obtain evidence for the proposed structure switching process (Figure 2B). In each experiment, the preannealed ATP Reporter A was incubated at 15 °C for 10 min, followed by a rapid temperature increase (within 1 min) from 15 °C to a designated temperature (37, 40, 45, or 55 °C), followed by a 50-minute incubation at each elevated temperature. Finally, the solution was rapidly cooled (within 1 min) to 22 °C and incubated at this temperature for 30 min.

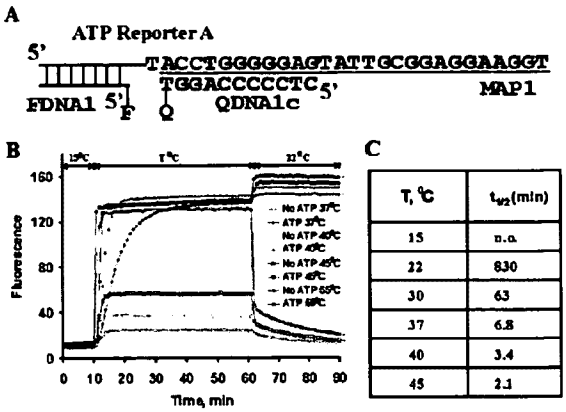


Figure 2. Evidence for structure switching. (A) DNA molecules used to assemble ATP Reporter A. (B) Examination of ATP Reporter A for structural transitions using temperature-changing experiments. Unfilled data points were for samples containing no ATP and filled data points were for ATP-containing solutions. Temperature settings are indicated on the top of the graph and in the embedded legends. The fluorescence intensity was measured every minute when the temperature of each solution was changed as follows: a 10-min incubation at 15 °C, followed by a temperature increase to 37, 40, 45, or 55 °C within 1 min and further incubation at the raised temperature for 50 min. Finally, each sample was cooled to 22 °C in 1 min and incubated at 22 °C for 30 more min. For the ATP-containing samples, 1 mM ATP was added to the preformed duplex DNA mixture stored at 4 °C and the resulting mixture was immediately examined. Other conditions are given in the experimental protocol section. (C) A quantitative description of temperature dependence of ATP reporter E. $t_{1/2}$ is the time required for the ATP reporter to reach the half-maximal fluorescence intensity at a given temperature.

In the absence of ATP, the reporter had a low and stable fluorescence intensity at 15 °C. When the temperature was raised from 15 °C to 37, 40, 45, or 55 °C, the intensity of the solution increased in a manner that was indicative of heat denaturation of the DNA duplex assembly. A higher incubation temperature resulted in a higher fluorescence intensity because less and less QDNA1c remained as part of a duplex assembly. At each temperature, a stable fluorescence intensity value was reestablished after a few minutes, indicating that the equilibrium between the amount of free QDNA1c and the amount of the QDNA1c bound in the DNA duplex assembly was reached. When the solution temperature was lowered to 22 °C, the fluorescence intensity dropped owing to the reassociation of some free QDNA1c molecules into the DNA duplex structure.

The introduction of 1 mM ATP into the DNA mixture (filled data points) did not cause a rapid increase in fluorescence intensity at 15 °C and 22 °C (i.e., room temperature; data not shown). However, when the temperature was raised from 15 °C to 37, 40, 45, or 55 °C, rapid intensity increases were observed. We used $t_{1/2}$ (the time required for the DNA solution to reach the half-maximal fluorescence intensity after the addition of 1 mM ATP at a designated temperature) to provide a quantitative measurement of the temperature dependence of the ATP-promoted intensity increase (Figure 2C). The $t_{1/2}$ at 22 °C was very large at 830 min; at 37 °C, $t_{1/2}$ was shortened to 6.8 min; when the temperature rose to 45 °C, the half-maximal intensity was reached in about 2 minutes. At temperature points other than 55 °C, the presence of ATP caused a marked difference in the increase of fluorescence intensity. The contrast between the intensity changes of the ATP-containing and ATP-lacking solutions was even sharper when the temperature was lowered from each of the higher temperature points to 22 °C, whereas the ATP-lacking solutions experienced a very significant decrease in fluorescence intensity, all the ATP-containing samples (including the one treated at 55 °C) registered a noticeable intensity gain.

The above observations are consistent with the structure switching mechanism shown in Figure 1A. Rapid structure switching did not occur at low temperatures (such as 15 °C) because most of the MAP1 molecules existed in the duplex form where the ATP binding site was partially occupied by QDNA1c. A rapid structural transition happened at the elevated temperatures because more QDNA1c molecules were forced to dissociate from the duplex assembly, and as a result, more free MAP molecules had their ATP-binding site freed for ATP binding. When the solution was cooled, whereas QDNA1c molecules naturally reannealed back onto the aptamer sequence in the absence of ATP, the formation of the ATP-aptamer complex in the ATP-containing solution prevented the reannealing.

The ATP-aptamer complex appeared to be very stable despite the presence of QDNA1c. This is evident from the observation that the fluorescence intensity stayed unchanged upon continuous incubation at 22 °C (from 62 to 90 min, Figure 2B). We also examined the fluorescence intensity of each solution after longer incubation times (up to 100 hours) and found virtually no reduction in fluorescence intensity (data not shown).

ATP Reporter A was then examined for sensing specificity (Supporting Figure 1). Although 1 mM ATP was able to produce ~90% of the maximum fluorescence signaling capability (as compared to the solution where the QDNA1c was omitted), CTP, UTP, or GTP at 1 mM were not able to induce significant intensity increases. The original ATP aptamer was known to bind dATP as well,[18] and indeed we found that the ATP reporter was able to bind to dATP (Supporting Figure 1). Furthermore, double mutations within the ATP binding site of MAP1 (mutant M1 and mutant M2) abolished the ATP-binding capability (Supporting Figure 2). All of these observations are consistent with the specific ligand-dependent structural transition mechanism depicted in Figure 1A.

Diverse Duplex Design. Our engineering strategy can be easily expanded to include a variety of modification choices and Figure 3A lists three more duplex configurations. ATP Reporters B and C are both bipartite systems involving the use of a fluorescein-dT (T1 and T15, respectively) as the fluorophore and a separate QDNA as the quencher. ATP Reporter D is another tripartite system where FDNA and QDNA were designed to bind two adjacent stretches of the unmodified DNA aptamer. The relevant QDNA and FDNA molecules were chosen for each configuration following the examination of thermal denaturation profiles of several constructs for each system (data not shown). All these four ATP reporters were tested for signaling capability and specificity (Figure 3B). Without exception, each of them was able to report specifically on the presence of ATP without false signaling for GTP (as well as CTP and UTP, data not shown). The ability to design alternative structure-switching configurations should help expand the usefulness of our strategy to allow the preparation of optimized signaling species for different aptamers.

Reporters for Real-time Sensing at Low Temperature. It is apparent that ATP Reporter A can only be used to perform real-time detection at elevated temperatures such as 40 °C or

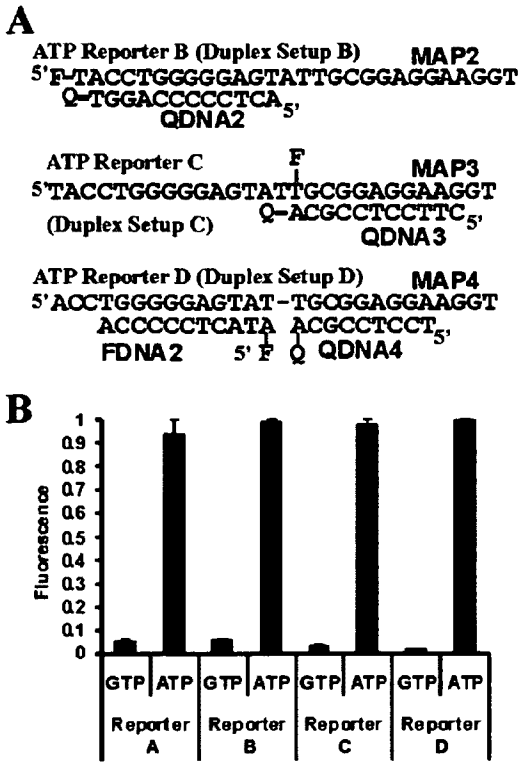


Figure 3. Different duplex configurations. (A) ATP Reporter B is a bipartite system consisting of the 5'-fluorescein labeled aptamer MAP2 and the 12-nt QDNA2. ATP Reporter C is another bipartite system made of aptamer MAP3 (internally labeled with fluorescein on T16) and the 11-nt QDNA3. ATP Reporter C is a tripartite system with the unmodified aptamer (MAP4), the 11-nt FDNA2 and 9-nt QDNA3. (B) Each reporter was examined without a target as well as in the presence of either ATP or GTP (in triplicate). The experiments were carried out as follows: A stock solution of a relevant MAP, QDNA and FDNA was combined with water, ATP or GTP and the resulting mixture was first incubated at 40 °C for 10 min, followed by incubation at 22 °C for 30 min before its fluorescence was measured. The final DNA concentrations were as follows: for ATP Reporter A, FDNA1 at 40 nM, MAP1 at 80 nM, QDNA1c at 120 nM; for ATP Reporter B, MAP2 at 40 nM, QDNA2 at 80 nM; for ATP Reporter C, MAP3 at 40 nM, QDNA3 at 80 nM; for ATP Reporter D, FDNA2 at 40 nM, MAP4 at 80 nM, QDNA4 at 120 nM. The final ATP or GTP concentration was 1 mM. The fluorescence intensities were normalized using the following equation: $(F - F_0)/(F_{max} - F_0)$, where F is the fluorescence intensity of each sample, F_0 and F_{max} are for the samples with the lowest and highest fluorescence intensities, respectively.

above but not at lower temperatures (Figure 2B). A system that is only able to perform real-time detection at elevated temperatures has two key drawbacks for practical sensing applications. First, our tripartite or bipartite structure-switching reporters have increased background intensity at higher temperatures, producing a smaller signaling magnitude upon the binding of target. Second, because most aptamers are created at room temperature, their binding affinities are usually at their best near this temperature, and therefore, the detection at higher temperatures may significantly reduce the sensitivity of the aptamer reporters. Thus, it is very desirable to engineer aptamer reporters that are capable of real-time sensing at room temperature.

We reasoned that the inability of ATP Reporter A to perform low-temperature real-time sensing was caused by the occupancy of a long stretch of aptamer sequence by QDNA, because the

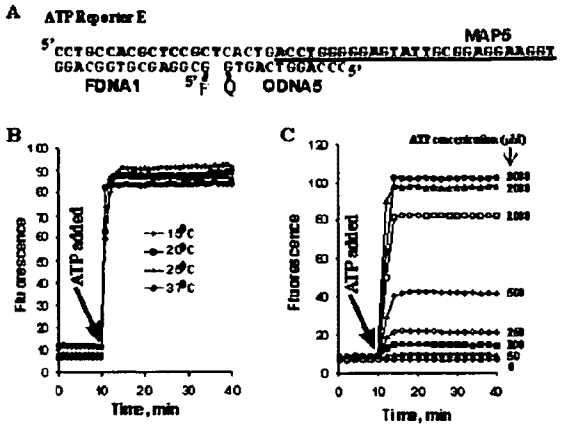


Figure 4. ATP reporter with real-time sensing capability at low temperature. (A) The DNA sequences used for the construction of ATP Reporter E. The modified aptamer contained the FDNA-binding domain (in dark blue), the original aptamer sequence (in black), and an inserted 5-nt domain (in medium blue) as part of the QDNA-binding domain. (B) Real-time sensing results. ATP reporter E was incubated in the absence of ATP for 10 min at an indicated temperature (15, 20, 25, or 37 °C), followed by the addition of ATP to 1 mM and a further incubation at the same temperature for 30 more min. (C) The real-time sensing capability of ATP Reporter E was examined as a function of ATP concentration at 20 °C. ATP was used at 0, 50, 100, 250, 500, 1000, 2000, and 3000 μM. The experiments were carried out the same way as in (B).

reporter was constructed with an 11-nt QDNA1c that was designed to block the first 11 nucleotides of the original 27-nt aptamer sequence. We hypothesized that if we could reduce the number of the blocked nucleotides in the aptamer sequence from 11 to a smaller number (such as 6 or 7), we might then be able to produce a low-temperature reporter. Because the reduction in the number of blocked nucleotides in the aptamer cannot be achieved simply by using a smaller QDNA (because this would produce a less stable duplex structure and a larger background signal), we decided to introduce additional nucleotides between the aptamer sequence and the FDNA-binding motif. Thus, we redesigned ATP Reporter A into the new tripartite system shown in Figure 4A (denoted ATP Reporter E) by inserting an arbitrary 5-nt sequence, CACGT, between the FDNA-binding domain and the aptamer motif. A 12-nt QDNA5 was used as the new quencher (it forms base pairs with both the five inserted nucleotides and the first seven nucleotides in the aptamer sequence).

ATP Reporter E was tested for real-time signaling at 15, 20, 25, and 37 °C and the data are shown in Figure 4B (the signaling DNA mixture was incubated at a designated temperature in the absence of ATP for 10 min, followed by the addition of 1 mM ATP and further incubation for 30 more min). ATP Reporter E was found to switch very quickly at all tested temperatures including 15 °C (the $t_{1/2}$ for ATP Reporter E at all these temperatures was all less than 1 min). In contrast, the ATP-promoted intensity increase of ATP Reporter A at 15 °C was too slow to determine a $t_{1/2}$ (Figure 2C). These data indicated that ATP Reporter E indeed had a much improved low-temperature real-time sensing capability.

ATP Reporter E also displayed a very large signaling magnitude (i.e., S/B, defined as the fluorescence intensity in the presence of ATP over that in the absence of any target) and the S/B values were found to be 14.1, 13.0, 10.4, and 7.1 at 15,

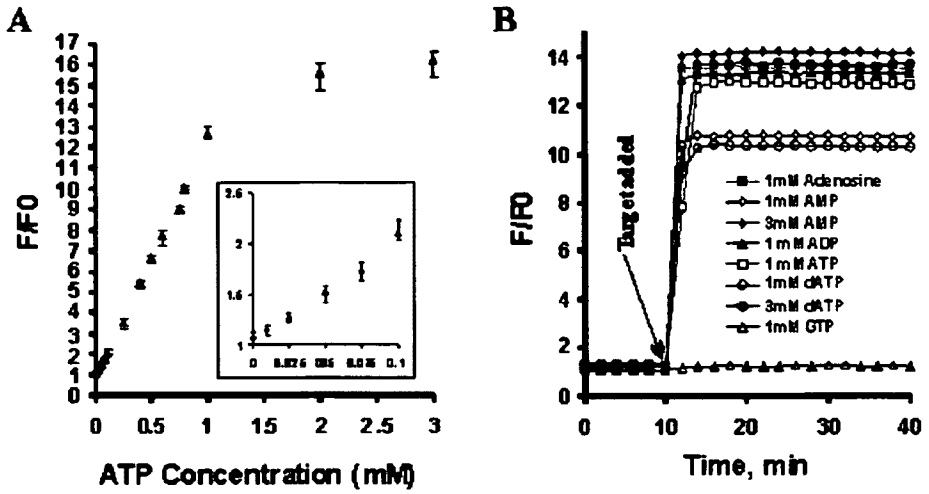


Figure 5. Target detection range and sensing specificity of ATP Reporter E. (A) The fluorescence intensity of ATP Reporter E was recorded in real-time at 20 °C with different concentrations of ATP (in triplicate) using the same experimental setting as in Figure 4B. The data obtained at the 20th minute were normalized using the following equation: F/F_0, where F is the fluorescence intensity of each sample, F_0 is for the sample with the lowest fluorescence intensity. ATP concentrations were 0, 10, 25, 50, 75, 100, 250, 400, 500, 600, 750, 800, 1000, 2000, and 3000 μM. Each data point represents the average value of three independent experiments with error bars indicated. (B) ATP Reporter E was examined for real-time sensing behavior in the presence of GTP (1 mM), ATP (1 mM), ADP (1 mM), AMP (1 mM and 3 mM), adenosine (1 mM) or dATP (1 mM and 3 mM). The data were normalized using the same equation as in (A), F/F_0, where F is the fluorescence intensity of each sample and F_0 is the initial reading for the reporter in the absence of any target.

20, 25, and 37 °C, respectively, upon the addition of 1 mM ATP. ATP Reporter E was also examined for intensity response to the increase of ATP concentration at 20 °C in real time (Figure 4C). Its signaling intensity increased linearly as the ATP concentration was raised between 0.01 and 1 mM (Figure 5A). This ATP reporter was also tested for target specificity (Figure 5B). Although the reporter registered a large signaling magnitude in the presence of 1 mM ATP, ADP, and adenosine, the addition of 1 mM CTP (data not shown), 1 mM UTP (data not shown) or GTP was not able to induce a change in the fluorescence signal. We found that 1 mM dATP and 1 mM AMP induced a smaller but still substantial fluorescence intensity increase (10-fold vs 13-fold for 1 mM ATP). We confirmed that the intensity reduction was not caused by the inaccuracy of target concentrations (the concentration of each target was carefully determined by the standard spectroscopic methods). We further found that the reduced signaling magnitude seen with 1 mM dATP and AMP was due to a shift in the saturating target concentration because the maximum fluorescence enhancement was achieved when 3 mM dATP or 3 mM AMP was used (Figure 4C). This later experiment suggests that the affinity of ATP Reporter E for AMP (and dATP) is noticeably lower than that for ATP, ADP, and adenosine. The above target specificity pattern is in good agreement with that observed for the original aptamer.[18] The observation of the reduced affinity for AMP by ATP Reporter E (in comparison to its affinity for ATP, ADP, and adenosine) is quite intriguing considering that these compounds have identical chemical structures except the 5′-phosphate groups.[21]

Signaling Complex for Protein Detection. To demonstrate the general applicability of the above design strategy, we engineered a new reporter by using a DNA aptamer previously isolated for thrombin binding.[20] We used a modified aptamer sequence, MAP6 (Figure 6A) that contained the same FDNA1-binding domain so that FDNA1 could again be used as the source of fluorophore. Seven nucleotides were inserted between the FDNA-binding domain and the aptamer sequence and the 12-nt QDNA6 was used as the quencher.

Evidence supportive of structure switching was also obtained using temperature-variation experiments similar to the ones shown in Figure 2B (data not shown). The real-time signaling ability of the thrombin reporter was assessed and the data are shown in Figure 6B. Rapid signal generation was observed upon the addition of thrombin at 30 °C ($t_{1/2}$ = 1.4 min) and 37 °C ($t_{1/2}$ = 1.2 min). The reporter also exhibited a fairly rapid change in signal at 25 °C ($t_{1/2}$ = 4.6 min). However, $t_{1/2}$ increasingly lengthened when the detection temperature was decreased further. For example, at room temperature (22 °C), the $t_{1/2}$ increased to 9.5 min. The need for a higher temperature for real-time sensing of thrombin (as compared to the ATP sensing with ATP Reporter E, which was capable of rapid real-time sensing even at 15 °C; see Figure 4C) might be caused by one of the following two factors (or combination of both): (1) the increased percentage of the blocked nucleotides for the thrombin reporter as 6 of the 15 nucleotides (40%) in the thrombin-binding aptamer domain were blocked by QDNA6 while only 7 of the 27 nucleotides (~26%) in the ATP-binding aptamer domain were occupied by QDNA5, and (2) the nonoptimal metal ion concentrations. The later factor might be important for the thrombin aptamer because it has a guanine-quartet based tertiary structure that is known to be sensitive to both metal ion identities and metal ion concentrations. A previous study has shown that although K^+ promotes the formation of stable aptamer-thrombin complex, other metal ions such as Mg^{2+} and Ca^{2+} do not support

(21) One possible explanation that we would like to offer is that the electronic environment of the 5′-end of adenosine and its phosphate analogues affects the binding affinity. More specifically, whereas the anti-ATP aptamer can tolerate one negative charge on the alpha-phosphate group of ATP and ADP, the two negative charges on the monophosphate group of AMP interferes the aptamer-target binding. Although interesting, this point is not pursued further as the focus of the current investigation is directed at the designs of structure-switching signaling aptamer.

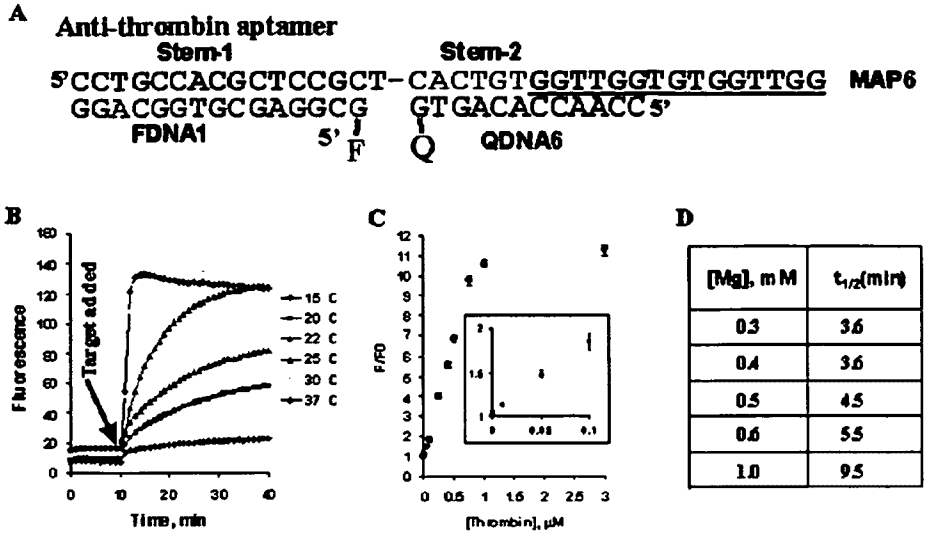


Figure 6. Structure-switching reporter for thrombin. (A) A previously isolated DNA aptamer for thrombin binding was configured into the tripartite signaling system using the similar design strategy employed for ATP Reporter E (see Figure 4A; the original thrombin-binding aptamer sequence is underlined). (B) Real-time sensing capability of the thrombin reporter was examined in a similar way to that shown in Figure 4A except that thrombin (1 μM) was used as the target and 5 mM KCl and 1 mM $MgCl_2$ as the metal ions. (C) The fluorescence intensity of the thrombin reporter was recorded in real time at 30 °C with different concentrations of thrombin (in triplicate) using the same experimental setting as in Figure 5A. The data point at the 20th minute for each sample was normalized using the following equation of F/F_0, where F is the fluorescence intensity of each sample, F_0 is for the sample with the lowest fluorescence intensity. The thrombin concentrations were 0, 10, 50, 100, 250, 400, 500, 750, 1000, and 3000 nM. Each data point represents the average value of three independent experiments with error bars indicated. (D) Influence on $t_{1/2}$ by magnesium concentration. $t_{1/2}$ is the time required for the thrombin reporter to reach the half-maximal fluorescence intensity at a given magnesium concentration at 22 °C.

the complex formation.[22] Our initial assaying mixture contained 1 mM $MgCl_2$ and 5 mM KCl and this condition was similar to that used in previous studies.[12,20] To determine whether the concentrations of potassium and magnesium ions might affect the real-time reporting capability of our thrombin reporter, we performed a series of real-time sensing measurements under different concentrations of KCl and $MgCl_2$. Although changing potassium concentration between 1 and 5 mM did not significantly affect the real-time sensing ability of the reporter (data not shown), lowering magnesium concentration enhanced the reporter's real-time detection capability at room temperature considerably (Figure 6D).

The signaling intensity of the thrombin reporter had a linear response to thrombin concentration over the range of 10−1000 nM (Figure 6C) and the maximum fluorescence enhancement reached nearly 12-fold. Once again, the target reporting was found to be very specific as other proteins including bovine serum albumin (BSA), and human factors Xa and IXa were not be able to generate fluorescence signals that were significantly above background (Supporting Figure 3). The successful engineering of two DNA aptamer reporters based on the same principle suggests that our engineering strategy indeed is general and can be easily adapted for the conversion of any DNA aptamer into a fluorescence reporter.

Discussion

Base-pairing is fundamental to nucleic acids and appropriate exploitation of this interaction (such as sense-antisense binding) has become a unique way to manipulate nucleic acid structure and function. Several recent reports have utilized antisense oligonucleotide binding to regulate nucleic acid functions such as inactivation of aptamer activity in vivo,[23] modulation of ribozyme and allosteric ribozyme activities in vitro.[24,25] In this study, we have shown that antisense oligonucleotide binding can also be explored as a general strategy to design fluorescent aptamers as real-time reporters to detect small molecules and proteins. We have demonstrated that such reporters can be easily engineered from existing DNA aptamers using a simple structure-switching/fluorescence-dequenching mechanism. Because our strategy exploits the common dual-structure-forming capability associated with each DNA aptamer, the methodology should be easy to generalize.

Two important factors need to be considered in the establishment of a generalizable method for converting aptamers into fluorescence reporters. First, the method should be broadly applicable for aptamers with different binding affinities. It is well documented that aptamers have typical affinities (K_d) in the nM range (or lower) for protein targets and in the μM range (or lower) for small-molecule targets. Therefore, we have chosen two representative aptamers for our study: the anti-ATP aptamer with a K_d of ~10 μM[18] and the anti-thrombin aptamer with a K_d of ~200 nM.[20] The successful generation of effective reporters using the same engineering principle but two different aptamers with substantial affinity difference indicates that our strategy is indeed generalizable for aptamers with a broad range of binding affinities. Second, the method should work well for

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aptamers of different sizes (typically ranging from a dozen nucleotides to several dozen nucleotides). Our design strategy, which is particularly flexible with variable choices of duplex configurations, is well suited to deal with both large and small aptamers. Our designing strategy does not rely on the availability of detailed tertiary or even secondary structure information. For example, the only prerequisite for constructing a reporter according to Duplex Setup A (or B) is the simple truncation of nonessential nucleotides from the 5′-end of the relevant original aptamer sequence so that the QDNA molecule can be designed to block the first few essential nucleotides. Therefore, our approach is very easy to generalize.

The use of QDNA to partially block the binding site of an aptamer was expected to reduce the affinity of an aptamer for its target. This is because the aptamer-target complex is not directly formed between the target and the free target-binding site of the aptamer but rather between the target and the aptamer that is partially occupied by QDNA. We found that ATP Reporter E had an apparent K_d (taken as the target concentration that induced half-maximal fluorescence intensity change) of ~600 μM for ATP (estimated with the data shown in Figure 5A), which is about 60-fold higher than that reported for the original ATP aptamer.[18] In contrast, the thrombin reporter had an apparent K_d of ~400 nM for the human thrombin (Figure 6B), representing only a 2-fold affinity reduction in comparison to the original thrombin aptamer.[20] These observations seem to suggest that our QDNA-binding-and-release strategy causes more reduction in the target-binding affinity to signaling reporters constructed with low-affinity aptamers than to those built with high-affinity aptamers. On the basis of this observation, we hypothesize that the magnitude of the apparent K_d increase (i.e., the reduction of affinity for a target) is dependent on the affinity of the original aptamer. For a high-affinity aptamer (e.g., the thrombin-binding aptamer), the strong binding interaction between the aptamer and the target competes favorably with the QDNA-aptamer duplex binding interaction. As a result, the use of QDNA has little effect on the affinity of the aptamer reporter. However, with a low-affinity aptamer (e.g., the ATP-binding aptamer), the relatively weak target-aptamer interaction does not compete favorably with the QDNA-aptamer interaction, and therefore higher target concentrations are required to shift the following equilibrium to the right

$$\text{FDNA} \cdot \text{MAP} \cdot \text{QDNA} + \text{Target} \rightleftharpoons \text{FDNA} \cdot \text{MAP} \cdot \text{Target} + \text{QDNA} \quad (1)$$

Consequently, the apparent K_d of the reporter is significantly increased. However, having studied only two signaling aptamers, there is not enough experimental evidence for us to make a general conclusion. More signaling aptamers will be examined in future experiments.

The structure-switching signaling aptamers also exhibit a large signaling magnitude. Both the anti-ATP and anti-thrombin reporters exhibited a maximum S/B value in excess of 10. The signaling aptamers retain the binding specificities reported for the original aptamers. For example, the original anti-ATP aptamer was reported to bind ATP, ADP, AMP, adenosine, and dATP but not CTP, GTP, or UTP;[17] the engineered ATP reporters displayed the same target-recognition properties. The structure-switching reporters can be used for real-time sensing although the temperature suitable for real-time detection is greatly influenced by the number of nucleotides in QDNA that block the nucleotides essential for the formation of the DNA-target complex. Although a structure-switching signaling aptamer constructed with a long QDNA that binds solely to the aptamer domain can only be used for real-time sensing at elevated temperatures (such as 37 °C), reporters built with the same size of QDNA that blocks fewer nucleotides in the aptamer domain can perform real-time detection at very low temperatures (as low as 15 °C). Metal ion concentrations may also influence a reporter's low-temperature sensing ability as we found that the lower Mg^{2+} concentration supported the low-temperature real-time sensing of the guanine quartet-containing anti-thrombin aptamer reporter.

DNA is easy to prepare and has exceptional chemical stability. DNA aptamers can be easily created by in vitro selection for binding to a diverse range of targets with both high binding affinity and specificity. The simplicity of our modification strategy and the effectiveness of resultant fluorescence reporters described herein should help promote the exploitation of DNA aptamers as molecular tools for the solution-based detection of biological cofactors, metabolites, proteins, and a variety of other ligands of interest.

Acknowledgment. We thank the members of the Li laboratory for helpful discussions, and Drs. David W. Andrews and John D. Brennan for helpful comments on the manuscript and for English corrections. This study was funded by the Canadian Institutes of Health Research, Natural Sciences and Engineering Research Council of Canada, and Canadian Foundation for Innovation. Y.L. holds a Canada Research Chair.

Supporting Information Available: Signaling specificity of ATP Reporter A (Supporting Figure 1), signaling behaviors of two reporters made of mutant anti-ATP aptamers (Supporting Figure 2), and signaling specificity of the thrombin reporter (Supporting Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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EXHIBIT D

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Communication

G-Quadruplex Formation of Thrombin-Binding Aptamer Detected by Electrospray Ionization Mass Spectrometry

M. Vairamani, and Michael L. Gross

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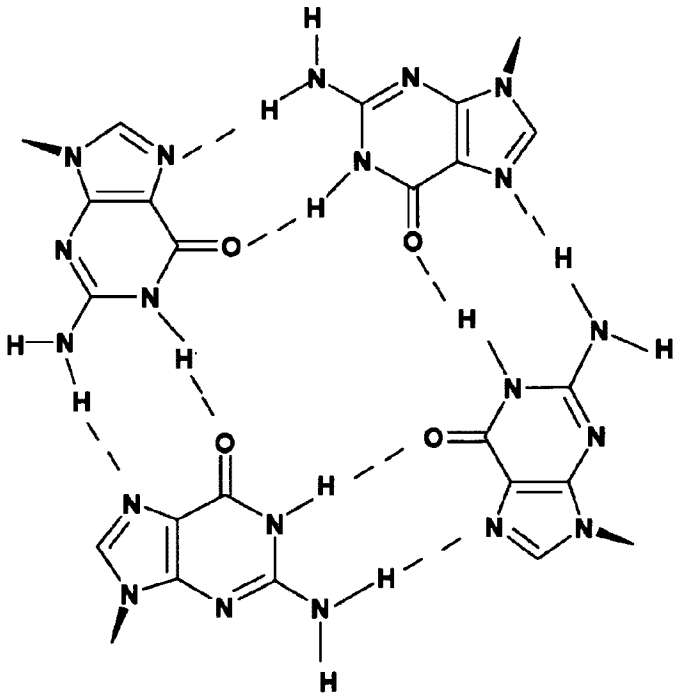

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G-Quadruplex Formation of Thrombin-Binding Aptamer Detected by Electrospray Ionization Mass Spectrometry

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The structure and properties of G-quadruplexes (Scheme 1) formed by oligodeoxynucleotides (ODNs)[1] are of interest because they exist in telomeric DNA and are stabilized by interactions with other molecules and ions, blocking telomeric activity and providing a basis for antitumor drug design.[2,3] The basic unit of a quadruplex is a quartet of guanine bases, stacked and held together by $\pi-\pi$ interactions. A recent crystal structure[4] shows that the loops holding together the G-quartets of telomere models are "propeller shaped", possibly explaining how they can be recognized by proteins.[4b] DNA aptamers also may contain G-quadruplex structures that allow them to bind to a desired target.[5] For example, the thrombin-binding, 15-mer aptamer d(GGTTGGTGTGGTTGG) specifically binds thrombin protein and inhibits thrombin-catalyzed fibrin-clot formation.[6]

Studies with circular dichroism, temperature-dependent UV spectroscopy, differential scanning calorimetry, isothermal titration calorimetry, and NMR[7-9] established the property of the 15-mer aptamer to form intramolecular G-quadruplex structures in the presence of various metal ions. There are only three reports, however, on mass spectrometric studies of G-quadruplexes in the gas phase: two on the observation of molecular ions of G-quadruplexes under electrospray ionization (ESI) conditions and one on their interaction with antitumor drugs.[10,11] Here we report for the first time evidence that ESI MS detects the formation of the G-quadruplex of the thrombin-binding aptamer in solution and establishes some of its properties in the gas phase, particularly its specific interaction with metal ions.

ESI of the thrombin-binding aptamer introduces into the gas phase $[M - nH]^{n-}$ ($n = 3-8$) ions.[12] In addition to the multiply deprotonated molecules, we expect ions in which H^+'s are replaced with alkali metal ions, if available. The key question is whether the metal-ion binding is specific. Many polar compounds undergo nonspecific, metal-ion binding upon desorption or spray ionization. Nonspecific addition would easily occur by the replacement of phosphate protons of the ODN. To determine if the metal-ion binding is specific, we selected d(AATTAATGTAATTAA), d(AGTTAGTGTAGTTAG), and d(GATTAGTGTGATTAG) as controls for which we replaced G bases with adenine. If the carbonyl groups of the guanine bases do coordinate to the metal ions to form a G-quadruplex, then those ODNs having that structure should form specific adducts to a greater extent than those that bind nonspecifically.

These control ODNs bind many Li^+, Na^+, K^+, and Cs^+ ions, as was seen by a cluster of ions in which addition of more than one metal ion occurs for lower charge states (3^- and 4^-) (data not shown), indicating that the binding is nonspecific. Addition of Rb^+ ion to the aptamer and to the control ODNs in various charge states is not significant.

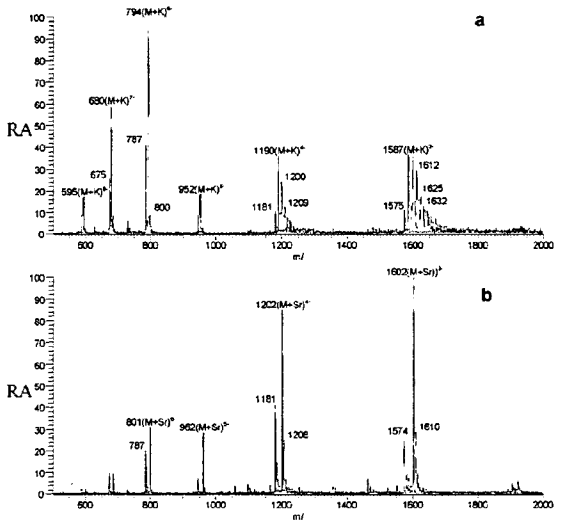
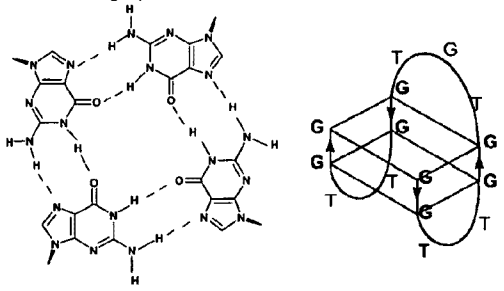


Figure 1. Negative-ion electrospray mass spectra of the aptamer in the presence of KCl (a) and $SrCl_2$ (b). RA is relative abundance.

Scheme 1. The Guanine Quadruplex (Left) and Schematic Diagram (Right) of the Intramolecular G-Quadruplex of the Thrombin-Binding Aptamer



In the case of K^+ (Figure 1a), however, we found evidence for enhanced adduct formation of the aptamer with one K^+; these adducts exist in the high charge states (6^- to 8^-). The control ODNs do not show such adduct formation. The addition of Na^+ to the aptamer may be partly specific, but the specificity is considerably lower than that exhibited by K^+ binding.

The divalent alkaline-earth metal ions Mg^{2+} and Ca^{2+} only interact weakly with the aptamer and the control ODNs. Sr^{2+} (Figure 1b) and Ba^{2+}, however, clearly form abundant adducts with the aptamer, but not with the controls, pointing to stability and indicating specific interactions in this aptamer's binding (data not shown). The extent of adduct formation is similar for Ba^{2+} and Sr^{2+}. Ion abundances show that adduct formation is more favorable

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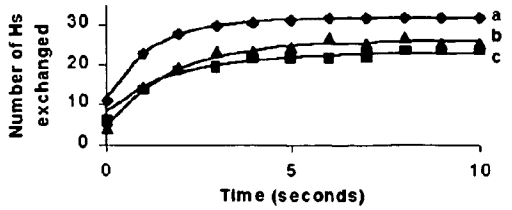

Figure 2. The time-dependent number of H's exchanged with CD_3OD in the gas phase for $(M - 7H)^{7-}$ (a), $(M - 8H + K)^{7-}$ (b), and $(M - 9H + Sr)^{7-}$ (c). The data are fit (solid curves) with two rel. rate constants of 1 and 10^5; P_{CD3OD} is unknown). The number of fast-exchanging H's is 12, 5, 8, whereas the slow-exchanging H's are 20, 21, 14 for (a), (b), (c), respectively. Only 50% of the experimental points are shown to avoid congestion.

for Sr^{2+} and Ba^{2+} ions than it is for K^+ (data not shown). These interpretations are consistent with those reached by using optical spectroscopy.[7]

Intrigued by these results, we studied in more detail the ability of the aptamer to bind Sr^{2+}. A 10-fold increase in the concentration of Sr^{2+} (to 50 μM) with respect to that of the aptamer (5 μM) does not lead to any significant additional binding of Sr^{2+}. The abundance of the $(M + Sr)^{n-}$ adduct increases nearly linearly from 0 to 15% when the methanol concentration goes from 0 to 25% (by vol). We suggest that a decrease in the dielectric constant of the solution causes Sr^{2+} to bind more effectively. We observe a similar effect when we used acetonitrile in place of methanol. The extent of adduct formation with Sr^{2+} at different charge states does not vary significantly with respect to the changes in the concentration of methanol or acetonitrile.

Others reported that Pb^{2+} and Mn^{2+} ions also bind to this aptamer in its quadruplex form.[9,13] Our experiments with $PbCl_2$ and $MnCl_2$ show that Pb^{2+} does form an adduct with the aptamer, although it is less abundant than those of Ba^{2+} and Sr^{2+}. We draw this conclusion because, in a mixture containing equimolar amounts of Ba^{2+}, Sr^{2+}, and Pb^{2+}, the adduct with Pb^{2+} is barely detectable. An adduct with Mn^{2+} is also undetectable. The control ODNs do not form detectable adducts with Pb^{2+} or Mn^{2+}. Others demonstrated, using NMR, that Tl^+ can replace K^+ in stabilizing the G-quadruplex structure, possibly because the ionic radii of the two ions are comparable.[14] We find that the binding with Tl^+, however, is insignificant.

Various measurements show that metal ions occupy the cavity between the two quadruplex structures formed by the eight guanine moieties and bind by coordination with the eight carbonyl groups of the guanines. Although the MS results presented thus far do not pinpoint the location of the metal ion, they do indicate that the interaction of the metal with the thrombin-binding aptamer is specific. The bonding in the gas phase is likely to be via the G-quadruplex form because Sr^{2+}, Pb^{2+}, Ba^{2+}, and K^+, which show enhanced binding, have similar ionic radii (1.26, 1.29, 1.42, and 1.51 Å, respectively).[15] When involved in eight-coordinate bonding, Ba^{2+}, Sr^{2+}, and K^+ do form quadruplex structures in solution.[7] The lack of a smooth correlation between the ionic radii and the extent of adduct formation for these four metal ions suggests that the size of the metal ions is not the only factor in stabilizing the intramolecular G-quadruplex structure. Nevertheless, ESI MS does offer another sensitive method for studying G quadruplex structures.

We then turned to a direct probe of the gas-phase structure, H/D exchange. When the thrombin-binding aptamer, with and without K^+ or Sr^{2+}, is stored in the ion trap as a function of time, the adducts undergo a time-dependent increase in H/D exchange with deuterated methanol (Figure 2). The CD_3OD was admitted with the helium buffer gas and held at a constant, low partial pressure in the trap. The number of active H's in the 7^- state of the aptamer is 41, which are the maximum that can exchange. At the longest time (10 s), 32 exchanges occurred for the aptamer, but approximately 6 and 9 fewer exchanges occurred for the potassium and the strontium adducts, respectively. This additional protection is evidence that the interactions with these metal ions stabilize additional H's because of the specific binding of the G-quadruplex in the gas phase. Further work to establish the G-quadruplex structure in this and in related systems, using the H/D-exchange probe, is in progress.

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(12) All mass spectrometry experiments were conducted using a Finnigan LCQ quadrupole ion-trap mass spectrometer under negative-ion ESI conditions. The spray voltage was 2.2 kV, and the capillary was at 200 °C. The aptamer and other ODNs were from IDT, Coralville, IA, and were used without further purification. The 10 μM samples were prepared in deionized water. Metal chlorides were used as sources for the metal ions except for Rb^+ and Cs^+ where iodides were used. Metal-salt solutions of 10 μM concentration were prepared in water:methanol (1:1) and 1% NH_3. Equal volumes of the ODN and the metal-salt solutions were mixed and then introduced into the ESI source with a syringe pump at 5 μL/min. For H/D exchange, 2.5 μM samples were used, and the sample solutions were sprayed at 1 μL/min. Experiments were in the MS/MS mode using "zero" for the activation amplitude and changing the activation time from 0.03 to 10 000 ms. Five scans were averaged at each time point, the averaged spectrum was smoothed, and the centroids of the peak distribution, which represented D uptake, were used for the modeling.

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EXHIBIT E

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A DNA Aptamer as a New Target-Specific Chiral Selector for HPLC

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Abstract: In this paper, a DNA aptamer, known to bind stereospecifically the D-enantiomer of an oligopeptide, i.e., arginine-vasopressin, was immobilized on a chromatographic support. The influence of various parameters (such as column temperature, eluent pH, and salt concentration) on the L- and D-peptide retention was investigated in order to provide information about the binding mechanism and then to define the utilization conditions of the aptamer column. The results suggest that dehydration at the binding interface, charge−charge interactions, and adaptive conformational transitions contribute to the specific D-peptide-aptamer complex formation. A very significant enantioselectivity was obtained in the optimal binding conditions, the D-peptide being strongly retained by the column while the L-peptide eluted in the void volume. A rapid baseline separation of peptide enantiomers was also achieved by modulating the elution conditions. Furthermore, it was established that the aptamer column was stable during an extended period of time. This work indicates that DNA aptamers, specifically selected against an enantiomer, could soon become very attractive as new target-specific chiral selectors for HPLC.

Introduction

The separation of enantiomers is an area of increasing interest in pharmaceutical or biological fields, as two enantiomers of the same chiral molecule may have completely different physiological behaviors. For example, it is well established that, frequently, one of the two enantiomers of a drug is pharmaceutically active, while the other one can be inactive or toxic. Thus, the industrials are becoming more and more interested in new separation methodology for both analysis and purification of optical antipodes. Intensive research efforts are carried out for the discovery of new peptidic drugs.[1] Notably, important papers have focused on the usefulness of peptides composed of D-amino acids (D-peptides) for the development and identification of potential drugs with resistance to proteolytic degradation.[2] Therefore, several papers have been reported for the chiral separation of oligopeptides.[3] High-performance liquid chromatography (HPLC) is one of the most suitable techniques for enantiomeric resolution at analytical (or preparative) levels because of its high efficiency, speed, reproducibility, and wide range of applications. During the last two decades, various types of chiral selectors have been introduced as chiral stationary phases (CSPs) in HPLC. The chiral selectors commonly used for the production of CSPs are amino acids,[4] proteins,[5] crown-ethers,[6] oligo- and polysaccharides,[7] or more recently macrocyclic antibiotics.[8] However, the conventional chiral stationary phases are not specifically designed for the enantiomers to separate, making screening of stationary phase libraries a necessary step in the method development. Therefore, research efforts have been carried out in order to obtain tailor-made chiral selectors. Two major approaches have been reported involving

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the production of imprinted polymers[9] or antibodies.[10] However, both these two tailor-made chiral stationary phases have some drawbacks. Columns packed with imprinted materials exhibit relatively reduced apparent enantioselectivity, peak asymmetry, and low sample load capacity due to a heterogeneous population of binding sites and a lack of recognition of a number of important compound classes.[9a,11] In addition, the use of antibodies as affinity stationary phases has constraints such as low surface loading, difficulty in tailoring the selectivity, and lack of antibodies for weakly immunogenic molecules.[12]

Nucleic acid aptamers are oligonucleotides, often single-stranded, with selective binding properties originating from in vitro selection experiments (SELEX methodology).[13] DNA or RNA aptamers have been identified for a broad spectrum of targets including metal ions,[14] organic dyes,[15] amino acids,[16] peptides,[17] proteins,[18] nucleotides,[19] and drugs.[20] The selectivity and affinity of aptamers have been recently used with much interest in flow cytometry,[21] sensors,[20a,22] ELISA-type assays,[23] capillary electrophoresis,[24] and affinity chromatography.[25] Aptamers present various advantages. They are produced by chemical synthesis in a short time, at low cost, with reproducibility and accuracy and at a high degree of purity. It is also easily possible to change their sequence in order to modulate their binding selectivity. In addition, they can be modified at precise locations by molecules such as biotin in order to allow attachment to the streptavidin surface. Finally, they are stable to long-term storage. In some cases of chiral compounds, the efficient monitoring of the selection procedure has allowed a very high specificity exemplified by the capability of the aptamer to bind stereoselectively the target. For example, Geiger et al.[26] reported the selection of RNA aptamers that bind L-arginine with submicromolar dissociation constants and high enantioselectivity. Other RNA aptamers have been isolated that can discriminate between L- and D-amino acids although showing relatively poor discrimination factors.[27] Williams et al.[28] produced a stereoselective DNA aptamer. This selected oligonucleotide binds with high affinity the D-enantiomer of an oligopeptide without significant affinity for the L-enantiomer. However, to the best of our knowledge, the enantioselective properties of RNA or DNA aptamers have never been exploited for an application in target-specific chiral separations.

[AVP]
Cys^1-Tyr^2-Phe^3-Gln^4-Asn^5-Cys^6-Pro^7-Arg^8-Gly^9-NH_2

Figure 1. Primary structure of arginine−vasopressin ([AVP]).

The aim of this paper was to examine the feasibility of using a DNA aptamer, characterized by its high enantioselective binding, as a specifically designed chiral selector. A biotinylated DNA aptamer, with stereoselective binding affinity for a test D-peptide (arginine-vasopressin), was immobilized on a streptavidin chromatographic support. The retention and separation of the D- and L-peptides on this novel CSP was investigated in relation to column temperature, pH, and ionic strength of the mobile phase. A mechanism for the chiral discrimination of vasopressin enantiomers was proposed, and the operating conditions for optimal enantiomeric separation were determined.

Experimental Methods

Reagents and Materials. L-Vasopressin (Figure 1) was obtained from Sigma Aldrich (Saint-Quentin, France). D-Vasopressin was synthesized from D-amino acids by Millegen (Toulouse, France) and purified by reversed-phase chromatography (C8 column: 4.6 × 30 mm with a particle diameter of 7 μm; eluent A: H_2O; eluent B: H_2O−acetonitrile 25−75 (v/v); gradient elution by varying the proportion of eluent B in the mobile phase from 2 to 80% in 50 min; flow rate: 1 mL/min; UV detection: 215 nm; injection volume: 100 μL; solute retention time: 13.56 min). The identity of oligopeptide was confirmed by ESI-MS (before cyclization: m/z 1086.6 ($[M + H]^+$); after cyclization: m/z 1084.6 ($[M + H]^+$)). Na_2HPO_4, NaH_2PO_4, KCl, and $MgCl_2$ were supplied by Prolabo (Paris, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. The 55-base DNA oligonucleotide (Figure 2) was 5′-biotinylated (Eurogentec, Herstal, Belgium).

Biotin phosphoramidite containing a 16-atom spacer arm based on triethylene glycol was used for the aptamer biotinylation. The biotinylated oligonucleotide was purified by gel electrophoresis (Eurogentec). The 2.1 × 30 mm POROS BA column (perfusion chromatography column with 20 μm flow-through particles) and the loading buffer (10 mM phosphate, 150 mM NaCl, pH = 7.2) were obtained from Applied Biosystems (Courtaboeuf, France). In this column, streptavidin was covalently bound to the particle surface.

Stationary Phase Preparation. Prior to immobilization, the biotinylated aptamer was renaturated by heating the oligonucleotide at 70 °C for 5 min in an aqueous buffer (20 mM phosphate buffer, 25 mM

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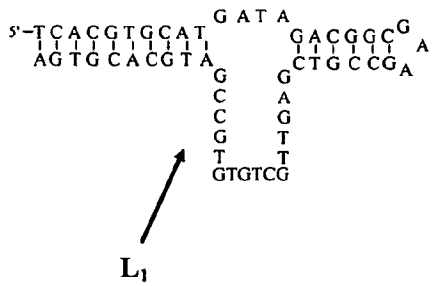

Figure 2. Sequence and secondary structure model of the 55-base DNA aptamer showing the asymmetric internal loop of 20 nucleotides (L_1) which is essential for the specific D-vasopressin enantiomer binding (from ref 28).

KCl, 1.5 mM $MgCl_2$ adjusted to pH 7.6) and left to stand at room temperature for 30 min. The POROS streptavidin column was equilibrated in loading buffer, by washing with ~20 mL. A 29 nmol sample of the modified aptamer was applied to the POROS streptavidin column using a pump fixed at a flow rate of 100 μL/min during 3 h, at room temperature. The column was washed with ~10 mL of loading buffer, and the column washes were added to the unbound DNA solution. The amount of oligonucleotide coupled to the chromatographic support was quantified by subtracting the UV absorbance at 280 nm of the unbound DNA from the initial solutions. When not used, the aptamer column was stored at 4 °C in the loading buffer.

Apparatus. The HPLC system consisted of a LC Shimadzu pump 10AT (Sarreguemines, France), a Shimadzu SIL-10AD auto injector, a Shimadzu SPD-10A UV−visible detector (λ = 195 nm), a Shimadzu SCL-10A system controller with Class-VP software (Shimadzu), and an Igloocil oven (Interchim).

Chromatographic Operating Conditions. The mobile phase consisted of 5 mM phosphate buffer and 3 mM $MgCl_2$. The phosphate buffer was prepared by mixing equimolar solutions of mono- and dibasic sodium phosphate to produce the desired eluent pH. The mobile phase pH ranged from 5.0 to 8.0, the column temperature from 0 to 25 °C, and the eluent KCl concentration from 25 to 100 mM. The mobile phase flow rates (50 or 150 μL/min) were low enough to exclude any split peak effect. To avoid the presence of significant nonlinear effects, the solute amount added onto the column corresponded to the smallest sample size allowing the detection of D-peptide in all operating conditions. Peptide solutions were prepared in the mobile phase at a concentration of 0.9 mM, and 100 nL was injected at least three times.

Determination of the Chromatographic Parameters. The solute retention on the aptamer stationary phase can be evaluated using the retention factor k:

$$k = \frac{t_R - t_0}{t_0} \tag{1}$$

where t_R is the retention time of the solute and t_0 is the column void time. To obtain the thermodynamic retention time, i.e., the accurate measure of solute retention, t_R was determined by calculating the first moment of the peak as previously described.[5a] The void time was determined using the mobile phase peak. The retention times and column void time were corrected for the extracolumn void time. It was assessed by injections of solute onto the chromatographic system when no column was present.

At infinite dilution, i.e., under linear elution conditions, and assuming that nonspecific interactions between solute and chromatographic support were negligible (see below), the retention factor can be related to the association constant between peptide and aptamer K as follows:

$$k = \sigma K \tag{2}$$

where σ is equal to the ratio of the active binding site number in the column (m_L) over the void volume of the chromatographic column (V_M).

The efficiency of the column, reflecting the band broadening, was characterized by estimating the reduced plate height h (the smaller the reduced plate height, the greater the efficiency):

$$h = \frac{L}{d_p N} \tag{3}$$

with

$$N = 5.54\left(\frac{t_R}{\delta}\right)^2 \tag{4}$$

where N is the number of theoretical plates (δ is the peak width at half-height), L is the column length, and d_p is the average particle diameter.

The asymmetry factor A_s, reflecting the peak distortion, was determined by calculating the ratio of the second (or right) part of the peak over the first (or early) part of the peak at 10% of the peak height.

Analysis of the Retention Data. The model equations were fitted to the retention factors of the solutes using the software Table Curve 2D (SPSS Science Software GmbH, Erkrath, Germany).

Results and Discussion

The chromatographic column used in this study consisted of a D-vasopressin-specific DNA aptamer immobilized on a highly porous polystyrene−divinylbenzene support via a biotin−streptavidin bridge. It has been previously established that this 55-base aptamer bound the D-enantiomer of peptide with an association constant of ~1.1 μM^{-1}, while no significant affinity for the L-enantiomer was observed.[28] This aptamer is characterized by an asymmetric internal loop of 20 nucleotides, which is essential for the specific D-vasopressin binding (see Figure 2). A 21 nmol sample of aptamer was immobilized for a bed volume of 100 μL. The maximum binding capacity of the POROS streptavidin media was approximately 12.5 nmol of biotinylated antibody per 100 μL.[29] This difference between oligonucleotide and antibody immobilization indicates that the aptamer can be bound more densely than the antibody due to the difference in the steric effects. Similar conclusions have been drawn by Deng et al.,[25a] who immobilized an adenosine-specific DNA aptamer on a streptavidin support, for the separation of adenosine and analogues by affinity chromatography. The analysis of the enantiomeric mixture was carried out in operating conditions similar to those originally used for the selection of the aptamer.[28] The mobile phase consisted of 5 mM phosphate buffer, 100 mM KCl, and 3.0 mM $MgCl_2$, pH 7.0. The column temperature was set at 20 °C. As shown in Figure 3, the D-peptide was retained by the affinity column, while the L-peptide eluted in the void volume.

This result indicates that the immobilized aptamer binds significantly the D-enantiomer without any significant binding to the L-enantiomer. To explore the mechanistic aspects of this chiral discrimination and to determine the optimal utilization of this aptamer column, experiments were performed under a variety of operating conditions (pH and ionic strength of the mobile phase and column temperature). The chromatographic results are presented below.

Bulk Mobile Phase pH Effects. Vasopressin has three ionizable groups: the N-terminal amino, the arginine guanidine,

(29) As indicated in the operating conditions supplied by Applied Biosystems.

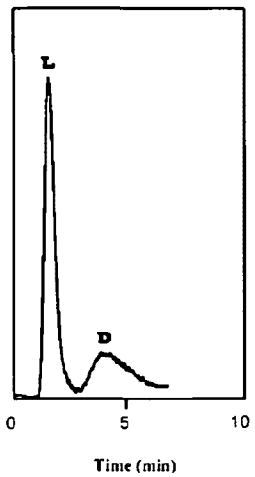

Figure 3. Separation of the vasopressin enantiomer pair (**L**: L-enantiomer; **D**: D-enantiomer) using the target-specific aptamer stationary phase. Column: 2.1 × 30 mm. Temperature: 20 °C. Mobile phase composition: 5 mM phosphate buffer, 100 mM KCl, 3 mM $MgCl_2$, pH 7.0. Flow rate: 150 μL/min. Injection: 100 nL at a concentration of 0.9 mM. UV detection at 195 nm.

and the tyrosine phenol groups. In a peptide, the mean values of pK_a for the arginine guanidine, tyrosine phenol, and cysteine N-terminal amino groups are ~12.5, ~10.5, and ~7.3, respectively.[30] Therefore, at eluent pH values lower than 8.5, arginine guanidine and tyrosine phenol groups are expected to be entirely protonated. On the other hand, the deprotonation of the N-terminal amino group is expected to start at lower eluent pH. By analyzing the pH effects on solute retention in the eluent pH range from 5.0 to 8.0, it was possible to evaluate a possible Coulomb interaction between the peptide N-terminal amino group and phosphate groups of DNA. The column temperature was 20 °C with a mobile phase consisting of 5 mM phosphate buffer, 100 mM KCl, and 3 mM $MgCl_2$. The L-enantiomer eluted in the void volume at all the mobile phase pH's. On the other hand, the D-enantiomer of vasopressin was significantly retained by the column over this eluent pH range. However, no significant variation of k_D was observed over the pH range as presented in Figure 4.

Such retention behavior is consistent with a binding mechanism in which the N-terminal amino group on the D-peptide is not essential to the interaction with the aptamer stationary phase.

Bulk Mobile Phase Salt Effects: The Polyelectrolyte Effect. The knowledge of the salt effect operative in the peptide–DNA interacting system could provide valuable information on the role of the Coulomb interactions in the association process. Previous papers have reported that relations derived from the Wyman concept constitute a valuable tool to describe the salt dependence on the solute retention in hydrophobic, electrostatic interaction and affinity chromatography.[31] The salt effects on the equilibrium constant K between the peptide and the aptamer can be modeled at a thermodynamic

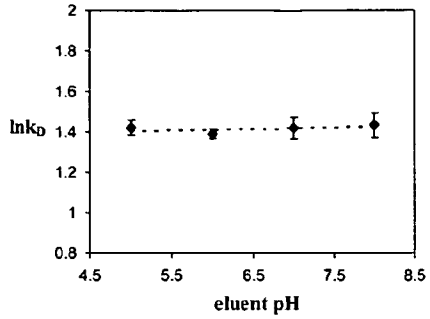

Figure 4. Plot of ln k versus eluent pH for D-peptide (k_D) using the target-specific aptamer stationary phase. pH range: 5.0–8.0. Column: 2.1 × 30 mm. Temperature: 20 °C. Mobile phase composition: 5 mM phosphate buffer, 100 mM KCl, 3 mM $MgCl_2$. Flow rate: 150 μL/min.

level in terms of the direct stoichiometric participation of ions (ν_x) and water (ν_w) in the association reaction. The dependence of K on the mean ionic activity a_x can be formulated as follows, via the linkage Wyman relations modified by Tanford:[32]

$$\frac{d(\ln K)}{d(\ln a_x)} = (\Delta\nu_x) - \frac{pm_x}{55.6}(\Delta\nu_w) \qquad (5)$$

where $(\Delta\nu_x)$ and $(\Delta\nu_w)$ are respectively the net number of salt ions and water displaced or bound in forming the peptide–DNA complex, m_x is the molal concentration of salt, and p is the total number of ions associated with the electrolyte. At low enough salt concentration, the consequences of water release are insignificant. So, the dependence of K on salt activity provides a measure of the net number of ions released or bound upon complex formation.[33] Assuming that replacing the ionic activity by salt concentration c_x introduces little error over the experimental salt concentration range, an approximate integrated form of the equation is obtained as previously reported:[33]

$$\ln K \cong \ln K_0 + (\Delta\nu_x) \ln c_x \qquad (6)$$

where K_0 is the binding constant in a hypothetical 1 M salt concentration reference state.

Using eqs 6 and 2 the following relation can be obtained:[31a]

$$\ln k \cong \ln k_0 + (\Delta\nu_x) \ln c_x \qquad (7)$$

where k_0 is the retention factor corresponding to K_0.

It is well established that, for the interactions between a cationic ligand and double-stranded or single-stranded DNA, a decrease in the equilibrium association constant is observed with increasing salt concentration.[34] Previous thermodynamic studies have shown that this salt dependence is mainly due to the release of counterions (cations) from the DNA phosphate backbone upon the complex formation.[33,34] The high electrostatic potential from the negatively charged backbone of the nucleic acid is responsible for an accumulation of cations in the vicinity of

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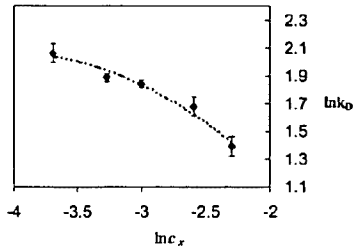

Figure 5. Plot of ln k versus ln c_x for D-peptide (k_D) using the target-specific aptamer stationary phase. KCl concentration (c_x) range: 25−100 mM. Column: 2.1 × 30 mm. Temperature: 20 °C. Mobile phase composition: 5 mM phosphate buffer, 3 mM $MgCl_2$, pH 6.0. Flow rate: 150 μL/min. (- - -) Theoretical curve obtained by fitting eq 9 to the experimental data.

the nucleic acid (polyelectrolyte nature of the nucleic acid). In fact, when a cationic ligand interacts with a nucleic acid, the neutralization of phosphate results in the release of the associated counterions. For the DNA of the length used here (55 bases), DNA behaves as a polyelectrolyte.[35] Therefore, Δv_x can provide an estimate of both the number of DNA phosphate charges neutralized in the interaction and the contribution of the polyelectrolyte effect to the cationic ligand−DNA complex.[33,34] Thus, the following relation can be obtained from eq 7:

$$\ln k \cong \ln k_0 - m'\Psi \ln c_x \tag{8}$$

where m' is the number of ion pairs and Ψ the degree of cation condensation per phosphate (assumed to be 0.76 for single-stranded DNA[34b]). In this approach, the presence of Mg^{2+} as potential competitive cation for phosphate groups has been neglected. Taking into account this effect, the following relation is described:[36]

$$\ln k \cong \ln k_0 - m'\Psi \ln c_x - m' \ln\left[0.5\left(1 + \sqrt{1 + \frac{4K_{Mg}c_{Mg}}{c_x^{2\Psi}}}\right)\right] \tag{9}$$

where c_{Mg} is the Mg^{2+} concentration in the medium and K_{Mg} the equilibrium constant at 1 M between Mg^{2+} and phosphates on DNA.

The analysis of the salt effects was carried out by measuring the retention time of L- and D-peptides in the eluent KCl concentration range from 25 to 100 mM at a column temperature of 20 °C. The mobile phase consisted of 5 mM phosphate buffer and 3 mM $MgCl_2$, pH 6.0. The retention factor of the D-peptide decreased with the mobile phase KCl concentration, while the L-peptide was always eluted in the void volume. Figure 5 shows the ln k versus ln c_x plot for the D-enantiomer at pH 6.0.

To assess if this retention factor change with increasing salt concentration was due to a variation in the binding capacity of the column, the concentration dependencies of the D-peptide retention were measured at different c_x.[37] No change in the number of binding sites was observed when the concentration of salt varied. These results demonstrate that Coulomb interactions participate in retaining the D-peptide. The m' value obtained from eq 9 by a nonlinear regression procedure was 1.0 ± 0.4 ($r^2 = 0.964$), suggesting that only one charge−charge interaction was involved for the D-enantiomer binding.

Column Temperature Effects and Determination of Thermodynamic Parameters. Valuable information about the processes driving the peptide chiral discrimination can be further gained by examining the temperature dependence on solute retention.[38] The temperature dependence of the retention factor is given by the following relation:

$$\ln k = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} + \ln \sigma \tag{10}$$

where ΔH and ΔS are respectively the enthalpy and entropy of transfer of solute from the mobile to the stationary phase, T is the absolute temperature, and R is the gas constant. If the stationary phase, peptide, and solvent properties are independent of temperature and ΔH and ΔS are temperature invariant, a linear van't Hoff plot is obtained. When ΔH and ΔS are temperature dependent, the following logarithmic equation can be given assuming invariance of heat capacity change ΔC_p with temperature:[39]

$$\ln k = \frac{\Delta C_p}{R}\left(\frac{T_H}{T} - \ln\frac{T_S}{T} - 1\right) + \ln \sigma \tag{11}$$

where T_H and T_S are reference temperatures at which ΔH and ΔS are nil. Enthalpy and entropy changes can be evaluated as follows:

$$\Delta H = \Delta C_p(T - T_H) \tag{12}$$

$$\Delta S = \Delta C_p \ln\left(\frac{T}{T_S}\right) \tag{13}$$

The analysis of the thermodynamics was carried out by measuring the D-peptide retention factor in the temperature range from 0 to 25 °C. The mobile phase consisted of 5 mM phosphate buffer and 3 mM $MgCl_2$, pH 6.0. The van't Hoff plot for the D-peptide exhibits a significant nonlinear behavior as shown in Figure 6.

The concentration dependencies of the solute retention factor were also measured at different temperature in order to assess if the nonlinearity of the van't Hoff plot was due to a variation in the number of active binding sites.[37] As reported above for the salt experiments, the binding capacity of the column was

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(37) Under moderate nonlinear conditions, Snyder and co-workers (*J. Chromatogr.* **1987**, *384*, 45) have established that the apparent solute retention factor k_a can be described by the following single function: $k_a = kf\{[k/(k + 1)]N^{1/2}Q_x/m_L\}$ where k and N are respectively the retention factor and the number of theoretical plates (at infinite dilution), m_L is the number of active sites, and Q_x is the amount of solute injected. In two different operating conditions (named 1 and 2) where $[k_1/(k_1 + 1)]N_1^{1/2} \approx [k_2/(k_2 + 1)]N_2^{1/2}$, the k_{a1}/k_{a2} ratio is expected to be invariant with Q_x if m_L is constant. Overloading experiments were carried out at the different salt or temperature conditions for which $N_1 \approx N_2$ and $[k_1/(k_1 + 1)] \approx [k_2/(k_2 + 1)]$ (for high k values with k_1 not much different from k_2). The k_a ratios were found to be invariant with the amount of solute injected, indicating that no changes in the m_L value occurred with varying salt or temperature.
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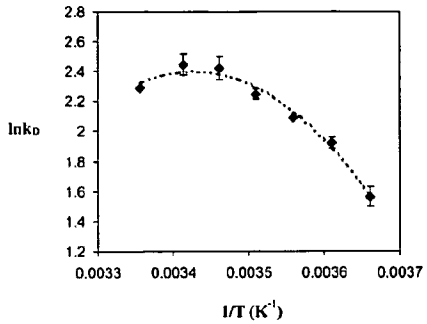

Figure 6. Van't Hoff plot for D-peptide (k_D) using the target-specific aptamer stationary phase. Temperature (T) range: 0−25 °C. Column: 2.1 × 30 mm. Mobile phase composition: 5 mM phosphate buffer, 3 mM $MgCl_2$, pH 6.0. Flow rate: 150 μL/min. (- - -) Theoretical curve obtained by fitting eq 11 to the experimental data.

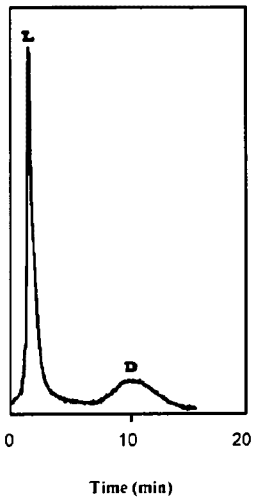

Figure 7. Separation of the vasopressin enantiomer pair (**L**: L-enantiomer; **D**: D-enantiomer) using the target-specific aptamer stationary phase. Column: 2.1 × 30 mm. Temperature: 20 °C. Mobile phase composition: 5 mM phosphate buffer, 3 mM $MgCl_2$, pH 6.0. Flow rate: 150 μL/min. Injection: 100 nL at a concentration of 0.9 mM. UV detection at 195 nm.

invariant when the temperature changed. Therefore, the nonlinearity of the van't Hoff plot is consistent with a large and negative heat capacity change. Figure 7 presents a chromatogram obtained by injection of an enantiomeric mixture at T equal to 20 °C.

From the nonlinear ln k versus $1/T$ plot, the thermodynamic parameters for D-peptide interaction with the aptamer were determined using eqs 11−13 (r^2 = 0.983). Table 1 shows the values of ΔC_p, T_H, T_S, ΔH, and ΔS.

For the determination of T_S and ΔS, the number of moles of immobilized aptamer was used as the m_L value assuming that the aptamer immobilized on the column was available for an interaction with the D-peptide. However, it is not always verified and the number of moles of active binding sites in an affinity protein-based column can be lower than the number of moles of ligand effectively immobilized. This is due to various factors such as steric hindrance, denaturation, or inefficient orientation.[40] For example, it has been shown that from ~10% to ~80% of the β-blocker sites,[40a] benzoin sites,[40b] and warfarin sites[40c] are

Table 1. Temperature Dependence of Thermodynamic Quantities Associated with the D-Peptide retention on the Aptamer Stationary Phase As Evaluated by Fitting Eq 11 to Experimental Data[a]

temp (°C)	ΔH (kJ/mol)	ΔS (J/mol·K)	ΔC_p (kJ/mol·K)	T_H (°C)	T_S (°C)
0	71.0 (2.3)	341.0 (10.0)			
4	55.9 (1.2)	286.1 (6.3)			
8	40.8 (0.08)	232.0 (1.6)			
12	25.7 (1.2)	178.6 (2.5)	−3.780 (0.293)	18.8 (0.9)	25.8 (1.4)
16	10.6 (2.4)	126.0 (6.5)			
20	−4.5 (3.5)	74.0 (10.5)			
25	−23.4 (5.0)	10.1 (15.1)			

[a] Standard deviations are in parentheses.

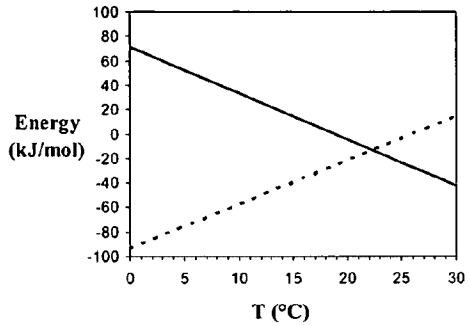

Figure 8. ΔH (—) and $-T\Delta S$ (- - -) plots vs column temperature (T) for D-peptide retention on the target-specific aptamer stationary phase. Column: 2.1 × 30 mm. Mobile phase composition: 5 mM phosphate buffer, 3 mM $MgCl_2$, pH 6.0. Flow rate: 150 μL/min.

active in various protein-based columns. Therefore, T_S and ΔS were also determined using an m_L value arbitrarily fixed to 10 nmol, representing ~50% of the number of moles of aptamer effectively immobilized. Very low changes in the T_S and ΔS values are observed.[41] For example, at 10 °C, the entropy change was only 3% higher than the ΔS value obtained using the number of moles of ligand effectively immobilized. So, neglecting these effects has no serious consequences on the interpretation of the thermodynamics. As a consequence of heat capacity change, the enthalpic and entropic contributions are strongly a function of temperature. Figure 8 shows the ΔH and $-T\Delta S$ values plotted as a function of the column temperature.

At low temperature, the binding of the D-enantiomer to the aptamer stationary phase is characterized by an unfavorable enthalpy term so that the association process is purely entropically driven. At about 25 °C, the enthalpy change of association was estimated to be approximately −20 kJ/mol with $-T\Delta S$ ~0 kJ/mol, indicating that the complex formation is enthalpically governed.

Possible Thermodynamic Origins of the D-Peptide Binding to the Immobilized Aptamer. L-Vasopressin does not exhibit any significant retention on the aptamer column under the various operating conditions. This means that possible Coulomb interactions, hydrophobic effects, or van der Waals interactions/hydrogen bonding with the chromatographic support or nonspecific regions of DNA are reduced and can be neglected in

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(41) For an m_L value of 10 nmol, T_S was found to be 26.3 °C.

this chromatographic system. Therefore, the D-peptide retention is exclusively dependent on the interactions with the specific binding pocket (the 20-base loop as indicated above). pH experiments suggest that the N-terminal amino group of the D-peptide is not involved in the binding at the stereospecific pocket. Such a behavior can be explained if it is considered that the aptamer has been selected against the target using D-vasopressin linked to the column through its N-terminal amino group. Moreover, no significant difference in the association constants was previously obtained for the D-vasopressin–aptamer or *N*-acetyl D-vasopressin–aptamer complexes.[28] As the salt experiments indicate that one charge–charge interaction occurs in the binding of D-peptide to the aptamer, it is strongly assumed that the arginine guanidine residue engages in a Coulomb interaction with one phosphate group at or near the specific binding loop. This is in agreement with previous data that showed that arginine guanidinium groups are frequently involved in the specific interaction between peptide/protein and nucleic acids. One example of the arginine-rich family of nucleic acid-binding proteins is the tat protein, which interacts specifically with TAR via the binding of a single arginine residue within a bulge situated within an RNA hairpin.[42] Furthermore, a study of Tao and Frankel described an RNA aptamer–arginine complex formation which involved both Coulomb interactions, hydrogen bonding, and stacking interactions.[43] Temperature experiments indicate the presence of a large and negative heat capacity change upon the D-peptide binding (Table 1). A large negative ΔC_p of association is a common feature of site-specific protein–nucleic acid interactions.[44] ΔC_p is classically related to hydration changes between two states of the system. Dehydration of nonpolar groups is a process accompanied by a major negative heat capacity change, while a positive heat capacity change is observed for the dehydration of polar surfaces.[45] Such a large, negative ΔC_p for D-peptide–aptamer interaction is consistent with a complex formation in which several contacts between nonpolar groups of two species are engaged. Consequently, it is strongly assumed that hydrophobic forces play a role in the D-peptide–aptamer complex formation. This effect explains, at least in part (see below), the entropically driven binding process observed at low temperature (Table 1 and Figure 8). However, it is well established that, at 25 °C, the transfer of hydrophobic compounds from water to nonpolar solvents ("pure" hydrophobic effect) is characterized by an enthalpy of formation close to zero and a large, positive entropy contribution.[46] At 25 °C, for the D-peptide–aptamer association, ΔH is approximately −20 kJ/mol (Table 1 and Figure 8). This means that van der Waals interactions and hydrogen bonding (both characterized by negative enthalpy changes at this temperature[46b,47]) are engaged at the complex interface. As $\Delta S \approx 0$ J/mol·K at 25 °C (Table 1 and Figure 8), it appears that there should be some source of negative entropy compensating the large, positive entropy of dehydration. The overall entropy change at 25 °C can be split up in the following way:[45a,47a,48]

$$\Delta S = \Delta S^{dehy} + \Delta S^{pe} + \Delta S^{tr/rt} + \Delta S^{conf} \approx 0 \qquad (14)$$

where favorable entropic changes include ΔS^{dehy} (entropy change due to dehydration effects) and ΔS^{pe} (entropy change due to the polyelectrolyte effect) and unfavorable contributions include $\Delta S^{tr/rt}$ (entropy change associated with the loss of translational and rotational degrees of freedom) and ΔS^{conf} (entropy change due to the loss of conformational freedom). The positive entropy contribution from counterion removal (polyelectrolyte effect) is estimated to be weak (20 J/mol·K).[49] In addition, direct experimental studies have shown that the entropy cost due to the reduction of translational and rotational degrees of freedom is small, i.e., $\Delta S^{tr/rt}$ is about −20 J/mol·K.[50] So, the major negative contribution to the entropy change would result mainly from the conformational entropy contribution, and an estimate of ΔS^{conf} can be obtained as follows:

$$\Delta S^{conf} \approx -\Delta S^{dehy} = -(\Delta S_{np}{}^{dehy} + \Delta S_{p}{}^{dehy}) \qquad (15)$$

where entropic effects of dehydration can result from the water release from both nonpolar ($\Delta S_{np}{}^{dehy}$) and polar ($\Delta S_{p}{}^{dehy}$) groups at the interface.[47a] Using the quantitative relationships previously developed for the analysis of various protein–DNA association thermodynamics, $\Delta S_{np}{}^{dehy}$ was estimated to be ~1300 J/mol·K at 25 °C.[51] Although $\Delta S_{p}{}^{dehy}$ cannot be evaluated, the polar group dehydration can only increase the positive component of the association entropy.[47a] Therefore, ΔS^{conf} is lower than −1300 J/mol·K. This unfavorable conformational entropy change could proceed from fixation of side chains at the interface and structural changes in the interacting molecules upon complex formation. The conformational entropy of fixing an average amino acid residue is about −40 J/mol·K.[45a,47a] So, the maximal entropy cost for the D-peptide binding is ca. −360 J/mol·K. This means that an other major negative entropy component comes from some structural changes in the interacting molecules upon complex formation. This expected behavior squares with the general feature of the association between peptide/protein and nucleic acid aptamer. Adaptive conformational transitions are classically associated with complex formation where both components are able to adjust their recognition surfaces in order to maximize complementarity through tightly packed contacts involving stacking, Coulomb interactions, and hydrogen bonding.[52]

Chromatographic Properties of the Target-Specific Aptamer Column. Enantioselectivity and Analysis Time. The main advantage of the target-specific aptamer column is that the enantioselectivity is very significant since the stationary phase binds only to the D-enantiomer; that is, the L-peptide interaction with the stationary phase is negligible whatever the operating conditions investigated. The separation factor is higher than the one classically observed with the imprinted chiral selectors[9a] and in the same order of magnitude as the one obtained using

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stereoselective monoclonal antibodies[10a] (for which the interaction of the nontarget enantiomer with immobilized antibody is also negligible). Here, it can be noted that the aptamer immobilization could affect the binding properties of the selector. However, such a possible behavior is expected to have a reduced effect since the chiral discrimination properties of the aptamer are maintained upon immobilization. So, the immobilization effects via streptavidin−biotin reaction allow the conservation of the stereoselective properties of DNA, and such an attachment procedure is useful for HPLC application. Both the separation of the vasopressin enantiomers and the analysis time can be easily modulated by varying the mobile phase salt concentration and the column temperature. Peptide enantiomers cannot be completely resolved using a high ionic strength mobile phase at a low column temperature. Baseline separation in a short time (around 7 min) is achieved at ambient temperature with 100 mM KCl in the mobile phase (Figure 3) or at a lower temperature without KCl in the eluent. At ambient temperature, a significant enhancement of the enantioselectivity, associated with a concomitant increase in the analysis time (around 15 min), is obtained when a low ionic strength mobile phase is used (Figure 7).

Band Broadening and Peak Asymmetry. Using eqs 3 and 4, the reduced plate height of the D-enantiomer was estimated to be comprised between 35 and 40 at a flow rate of 150 μL/min. As a comparison, the reduced plate height observed for L-phenylalanine anilide (the more retained enantiomer) on an imprinted chiral stationary phase varied from 35 to 150 with flow rate increasing.[9c] On the antibody-based chiral stationary phase, the h values obtained for the more retained enantiomer of various amino acids were comprised between 20 and more than 200 in relation to the flow rate and the compound type.[53] In addition, the D-peptide peak distortion was reflected by an asymmetry factor around 1.5 (ideal A_s is 1). When only one type of site is involved in the solute binding to the stationary phase as expected for the immobilized aptamer, broad and unsymmetrical peaks are the consequence of slow mass transfer kinetics (homogeneous kinetics tailing).[54] Several recent studies have demonstrated that target−aptamer complex formations are characterized by small rate constants,[55] as expected for a two-step association process that includes a rapid bimolecular association followed by rate-limiting slow structural changes which mediate the binding surface complementarity.[55c] So, no doubt that slow association−dissociation kinetics contribute significantly to the peak broadening and asymmetry that are observed with the aptamer column. Efficiency as well as peak shape can be significantly improved by increasing the Stanton number.[54] For example, the h value for the D-enantiomer was around 15 and $A_s \approx 1.2$ at a lower flow rate of 50 μL/min.

Column Stability. The column stability was evaluated by comparing the D-peptide retention factor before and after more than five months in the same conditions. No significant change in retention time was observed. This demonstrates the stability of the aptamer column during an extended period of time.

Concluding Remarks

In this paper, we report for the first time the use of an immobilized DNA aptamer as a new target-specific chiral stationary phase for high-performance liquid chromatography. Immobilized DNA aptamers could soon become very attractive chiral stationary phases specifically designed against an enantiomer since experiments show high stereospecificity, valuable binding capacity, and stability during an extended period of time. In addition, this work shows that an aptamer immobilized on a chromatographic support constitutes a valuable tool for examining the mechanistic aspects of the target−DNA complex formation. More overall, this new type of chiral selector could find applications in various other fields of chemistry such as enantioselective solid phase extraction, binding assays, and sensors. Further experiments are now in progress in our laboratory in order to select a stereoselective DNA aptamer against other enantiomeric compounds such as amino acids.

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JA034483T

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EXHIBIT F

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Article

Folding of the Thrombin Aptamer into a G-Quadruplex with Sr: Stability, Heat, and Hydration

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Folding of the Thrombin Aptamer into a G-Quadruplex with Sr^{2+}: Stability, Heat, and Hydration

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Abstract: It has been shown that the DNA aptamer d($G_2T_2G_2TGTG_2T_2G_2$) adopts an intramolecular G-quadruplex structure in the presence of K^+. Its affinity for trombin has been associated with the inhibition of thrombin-catalyzed fibrin clot formation. In this work, we used a combination of spectroscopy, calorimetry, density, and ultrasound techniques to determine the spectral characteristics, thermodynamics, and hydration effects for the formation of G-quadruplexes with a variety of monovalent and divalent metal ions. The formation of cation−aptamer complexes is relatively fast and highly reproducible. The comparison of their CD spectra and melting profiles as a function of strand concentration shows that K^+, Rb^+, NH_4^+, Sr^{2+}, and Ba^{2+} form intramolecular cation−aptamer complexes with transition temperatures above 25 °C. However, the cations Li^+, Na^+, Cs^+, Mg^{2+}, and Ca^{2+} form weaker complexes at very low temperatures. This is consistent with the observation that metal ions with ionic radii in the range 1.3−1.5 Å fit well within the two G-quartets of the complex, while the other cations cannot. The comparison of thermodynamic unfolding profiles of the Sr^{2+}−aptamer and K^+−aptamer complexes shows that the Sr^{2+}−aptamer complex is more stable, by ~18 °C, and unfolds with a lower endothermic heat of 8.3 kcal/mol. This is in excellent agreement with the exothermic heats of −16.8 kcal/mol and −25.7 kcal/mol for the binding of Sr^{2+} and K^+ to the aptamer, respectively. Furthermore, volume and compressibility parameters of cation binding show hydration effects resulting mainly from two contributions: the dehydration of both cation and guanine atomic groups and water uptake upon the folding of a single-strand into a G- quadruplex structure.

Introduction

The discovery of chromosomal sequences has led scientists to investigate the formation of unusual DNA structures.[1] Examples include telomere sequences, which are required to stabilize the ends of chromosomes[2] for the proper replication and segregation of eukaryotic chromosomes.[3] These telomere ends of 12−16 bases usually have G and T repeats in one strand that overhangs at the 3′ end.[4] Model telomere DNA sequences form a variety of tetraplex structures stabilized by cyclic hydrogen bonding of four guanines, forming G-quartets in the presence of univalent metal ions such as Na^+ or K^+.[5] G-quartets are also found commonly in DNA aptamers, which have been identified by some selection process to bind specific targets.[6] G-quadruplexes are remarkably stable both kinetically and thermodynamically[7−9] because of the tight association of cations with guanine residues.[10] The cations are coordinated to guanine O6 carbonyl groups between the planes of neighboring quartets. This provides a rationale for the observation that K^+ stabilizes such structures much more strongly than other univalent cations. Despite extensive investigations on G-quadruplexes, there is still a need to further our understanding of the role of cations in terms of their size, charge, and thermodynamic and hydration properties.

In this work, we use the oligonucleotide d($G_2T_2G_2TGTG_2T_2G_2$), which forms an intramolecular complex with K^+, as shown in Figure 1.[11] The complex is stabilized by two G-quartets connected by a TGT loop at the center and two T_2 loops.[11,12] This DNA aptamer binds thrombin with high-affinity and inhibits the thrombin-catalyzed fibrin clot formation.[13,14] Circular dichroism and melting techniques were employed to investigate the interaction of monovalent and divalent cations with this aptamer. In the presence of Rb^+, NH_4^+, Sr^{2+}, or Ba^{2+}, the oligonucleotide folds into stable intramolecular G-quadruplexes, similar to the one seen in the presence of K^+. We have further

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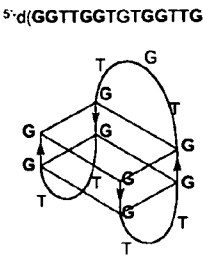

Figure 1. Sequence of the thrombin aptamer and its schematic structure.

characterized the thermodynamics and hydration contributions for the formation of G-quadruplexes with K^+ and Sr^{2+}. The Sr^{2+}−aptamer complex unfolds with a higher transition temperature and lower endothermic heat, but its favorable formation (in terms of $\Delta G°$) is comparable to that of the K^+−aptamer complex. Furthermore, the hydration effects for the formation of these cation−aptamer complexes are explained in terms of two contributions: the release of electrostricted water from the cations and guanine O6 atomic groups and the immobilization of structural water by the G-quadruplex from folding a single strand.

Materials and Methods

Materials. The oligonucleotide $d(G_2T_2G_2TGTG_2T_2G_2)$ was synthesized by the Core Synthetic Facility of the Eppley Research Institute at UNMC, HPLC purified, and desalted by column chromatography using G-10 columns. The concentration of oligomer solutions was determined at 260 nm and 80 °C using a molar extinction coefficient of 146 $mM^{-1}cm^{-1}$ (in strands). This value was calculated by extrapolation of the tabulated values of the dimers and monomer bases[15] at 25 °C to high temperatures using procedures reported earlier.[16] Reagent grade inorganic salts were purchased from either Fisher or Sigma and were used without further purification. All measurements were performed in a buffer solution consisting of 10 mM Cs−Hepes at pH 7.5; the ionic strength was adjusted by the addition of the appropriate salt.

Circular Dichroism (CD). The conformation of the cation−aptamer complexes was derived by simple inspection of their CD spectra. The CD spectra were obtained at several temperatures using an Aviv Model-202SF spectrometer (Lakewood, NJ) equipped with a peltier system for temperature control. Typically, a solution of oligonucleotide in the Cs^+ form was titrated with the appropriate cation solution, by stepwise addition of 3−5 μL aliquots of this solution until no further changes in the spectrum took place. Quartz cells with 1 cm or 0.5 mm path lengths were used to confirm that the conformation of complexes remains unchanged as a function of aptamer concentration, especially at the higher concentrations used in ultrasonic and volumetric experiments.

Temperature-Dependent UV Spectroscopy. Absorbance versus temperature profiles (melting curves) were measured for each complex at two wavelengths, 260 and 297 nm, with a thermoelectrically controlled Perkin-Elmer Lambda-10 spectrophotometer as a function of oligomer concentration. The temperature was scanned at a heating rate of 0.5 °C/min. These melting curves allow us to measure transition temperatures, T_M, which are the midpoint temperatures of the order−disorder transition of the complexes, and van't Hoff enthalpies, ΔH_{vH}, from analysis of the shape of the melting curves. In this analysis, a two-state approximation is used as reported earlier.[17] The formation of intramolecular complexes was assessed from the independency of T_M as a function of strand concentration.

High Sensitivity Differential Scanning Calorimetry (DSC). A MicroCal (Northampton, MA) VP-DSC differential scanning calorimeter is used to measure the total heat required for the unfolding of the cation−aptamer complexes. Complete thermodynamic profiles can be obtained from a single differential scanning calorimetric experiment using the following relationships:[17] $\Delta H_{cal}(T) = \int \Delta C_p \, dT$, $\Delta S(T) = \int (\Delta C_p/T) \, dT$, and $\Delta G(T) = \int \Delta C_p \, dT - T\int (\Delta C_p/T) \, dT$, where ΔC_p is the anomalous heat capacity during the transition. The assumption is made that no heat capacity effects take place between the initial and final states. In addition, van't Hoff enthalpies, ΔH_{vH}, can be evaluated from the half-width of the ΔC_p versus T (or ΔC_p versus $1/T$) experimental curves using the two-state approximation. The comparison of model-independent enthalpies ΔH_{cal} with ΔH_{vH} enthalpies allows us to examine if the transition takes place in a cooperative two-state fashion and whether intermediate states and/or aggregate states are present.[17,18]

Isothermal Titration Calorimetry. A Microcal (Northampton, MA) Omega instrument was used to measure the heat evolved during complex formation as a function of the amount of titrant from the mixing of cation and aptamer solutions. Typically, 5 μL aliquots of aptamer solution (210 μM in strands) are used to titrate the appropriate cation solution with a concentration of 50 μM (monovalents) or 10 μM (divalents). In the reverse titrations, 5−10 μL aliquots of the cation solutions were used to titrate a 36 μM aptamer solution. These experiments were geared to measure only binding heats, ΔH_{ITC}, from integrating and averaging the peaks of the initial injections, because the solute concentrations used in the reacting cell were greater than the inverse of the binding constant. The instrument was calibrated with a known electrical pulse, and its overall sensitivity is ~1 μcal.

Measurement of Hydration Parameters. The density, ρ, of all solutions was measured with an Anton Paar DMA-602 densimeter (Graz, Austria) with two microcells. The molar volume change, ΔV, accompanying the interaction of a cation with the aptamer is calculated with the equation $\Delta V = M_{complex}/\rho_{complex} - (M_{cation}/\rho_{cation} + M_{aptamer}/\rho_{aptamer})/(M_{cation}/\rho_{cation})C$, where ρ_i and M_i are the density and mass of the solution for each participating species, respectively, and C is the molarity of the cation solution used as the limiting reagent. All solutions were prepared by weight using a Mettler microbalance.

Ultrasonic velocity measurements were made with a home-built instrument in the frequency range 7−8 MHz.[19,20] The molar increment of ultrasonic velocity, A, is defined by the relationship $A = (U - U_o)/(U_oC)$, where U and U_o are the ultrasonic velocities of the solution and solvent, respectively. The changes in the molar increment of ultrasonic velocity, ΔA, are calculated with the relationship $\Delta A = A_{complex} - A_{cation}$. The change in the molar adiabatic compressibility, ΔK_S, is determined from ΔA and ΔV using the relationship $\Delta K_S = 2\beta_o(\Delta V - \Delta A)$, where β_o is the adiabatic compressibility coefficient of the solvent. The density and ultrasonic velocity measurements were done at 20 °C (±0.001 °C). Special precautions were taken in these experiments to prevent sample evaporation.

Hydration Contributions to the Volume and Compressibility Effects. The molar volume, ΦV, and molar adiabatic compressibility, ΦK_S, for a solute in a dilute solution are based on the following relationships:[21] $\Phi V = V_m + \Delta V_h$ and $\Phi K_S = K_m + \Delta K_h$. The V_m term is the molecular volume of the solute, while K_m is the molecular compressibility of this volume inaccessible to the surrounding solvent. The ΔV_h and ΔK_h terms are the hydration contributions. These contributions correspond to the changes in volume, and compressibility, of water around the solute molecule resulting from solute−water interactions and the void volume, or compressibility of this volume, between the solute molecule and of the surrounding water, respectively. The values of V_m and K_m for oligonucleotides, without significant inner cavities, are considered to remain constant during the course of a titration with cations.[22] The K_m term is actually small relative to the

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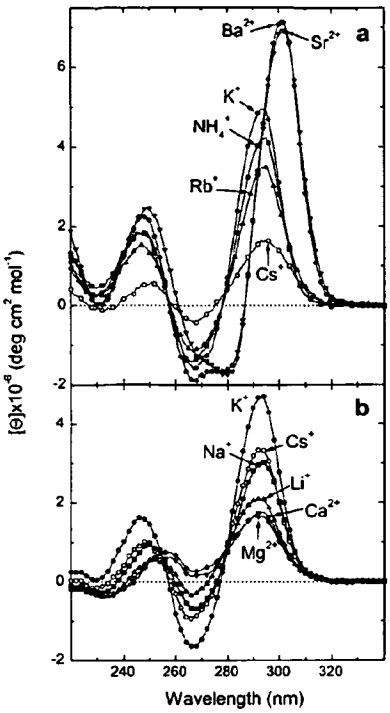

Figure 2. Circular dichroism spectra of d(GGTTGGTGTGGTTGG) with different cations in 10 mM Cs−Hepes buffer at pH 7.5 and at the following temperatures: 20 °C (a) and 2 °C (b). The concentrations used are as follows: 76 μM (oligonucleotide), 50 mM (monovalent cations), and 10 mM (divalent cations). The K^+ curves are included in both panels for proper comparisons.

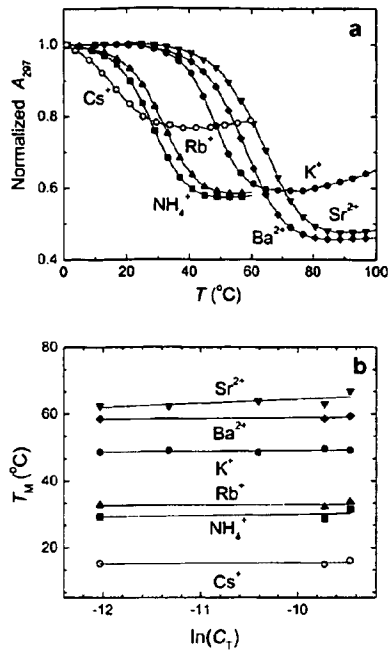

Figure 3. Optical unfolding of cation−aptamer complexes. (a) Typical UV melting curves in the presence of Cs^+, NH_4^+, Rb^+, K^+, Ba^{2+}, and Sr^{2+}, solution conditions as indicated in Figure 2. (b) Plots of the T_M dependence on strand concentration for each of the above cations.

hydration term;[22] therefore, the changes in ΦV and ΦK_S are simply a reflection of their hydration changes, that is, $\Delta V = \Delta\Delta V_h$ and $\Delta K_S = \Delta\Delta K_h$.

Results and Discussion

Circular Dichroism Spectra and Conformation of Cation−Aptamer Complexes. CD spectroscopy has proven to be a sensitive technique for determining the conformation of telomere model sequences. In this work, CD is used to determine the overall conformation of each cation−aptamer complex and its spectral characteristics at several temperatures. This was done in titration experiments of the oligomer in the Cs^+ form with each of the following cations: Li^+, Na^+, K^+, Rb^+, Cs^+, NH_4^+, Mg^{2+}, Ba^{2+}, Ca^{2+}, and Sr^{2+}. The CD spectra of the oligonucleotide saturated with each cation are shown at 20 °C and 2 °C in Figure 2a,b, respectively. The particular temperature for a given cation−aptamer complex corresponds to a temperature at which the equilibrium is shifted toward complex formation, as seen in the UV unfolding curves of the following section. The K^+ and Cs^+ curves are included in both plots for comparative purposes. Instead of the nearly conservative spectrum of the B-form with a crossover at 265 nm, all complexes have a nonconservative spectrum with a positive band centered at wavelengths ~290−300 nm, which characterizes the formation of antiparallel G-quartets.[23] The overall shape of the spectra is similar, but the magnitude and location of the positive and negative bands varies with the nature of the cation. Figure 2 reveals two types of CD spectra with distinctive characteristics of the positive band at longer wavelengths. The first type with Sr^{2+} and Ba^{2+}shows a large band of equal magnitude centered at 300 nm, and the overall spectra for these two cations are almost superimposable. The positive band in the second type is centered at 292 nm, and its magnitude varies in the following order: $K^+ > NH_4^+ > Rb^+ > Cs^+ > Na^+ > Li^+ > Ca^2 > Mg^{2+}$. Thus, their CD spectra have a similar shape, and the magnitude of the positive band at 292 nm changes gradually. The overlay of the spectra shows an isoelliptic point at 280 nm, which indicates the formation of a similar type of complex. At 20 °C, the magnitude of the positive band of the Cs^+−aptamer complex is only half the magnitude of the one at 2 °C, while for the K^+ complex the magnitude of this band remains the same. This shows that the Cs^+−aptamer complex has unfolded to some extent. The overall spectral differences between the complexes with these monovalent and divalent ions may be attributed to differences in the coordination number of the metal ion, partial formation of complexes with some cations and/or overall tightness of each complex. Electrostatic contributions may play a role in how tightly each complex is formed, which in turn depends on both the cationic charge and ionic size.

Unfolding of Complexes. UV melting curves at 297 nm of the aptamer complexes in cation solutions with similar ionic strength are shown in Figure 3a. The aptamer complexes with K^+, Rb^+, NH_4^+, Ba^{2+}, and Sr^{2+} unfold in monophasic transitions with large hypochromicities of 40−50%. The T_M values for the helix−coil transition of these complexes follow the order Sr^{2+} (63.1 °C) > Ba^{2+} > K^+ (48.7 °C) ≫ Rb^+ ~ NH_4^+ (29 °C). On the other hand, the Cs^+−aptamer complex melted with a T_M of 15 °C and a much smaller hypochomicity of ~23% in this short

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Table 1. Enthalpies for the Unfolding and Formation of Cation–Aptamer Complexes[a]

	UV	DSC		ITC	
cation	ΔH_{vH}	ΔH_{cal}	ΔH_{vH}	ΔH_{ITC}, 10 °C	ΔH_{ITC}, 20 °C
K^+	39	25.6	36	−19.2	−24.5 (−26.0)
Rb^+	35	21.6	31	−16.4	−19.6
Cs^+	24	6.5	29		
NH_4^+	33	20.9	31	−13.2	−18.5
Sr^{2+}	33	17.3	35	−10.2	−16.7 (−16.9)
Ba^{2+}	34	19.1	36	−10.3	−18.5

[a] All values determined in 10 mM Cs–Hepes buffer containing 50 mM KCl or 10 mM $SrCl_2$, at pH 7.5, and reported in kcal/mol. Experimental errors are given in parentheses: ΔH_{vH} (±15%) ΔH_{cal} (±5%), ΔH_{ITC} (±4%).

temperature range, as shown in Figure 3a. It should be noted that the transition of the Cs^+ complex is ~70% completed at 20 °C, in good agreement with the changes in ellipticity at these temperatures. The transitions with Na^+, Mg^{2+}, or Ca^{2+} (data not shown) were similar to the Cs^+ transition, while a Li^+ transition was not observed. The UV melting curves at 260 nm (data not shown) yielded T_M's in good agreement with the ones obtained at 297 nm. However, the changes in absorbance with temperature are different; for instance, the K^+ melting curve yielded a 6% hypochromicity, while the Sr^{2+} curve had a 6% hyperchromicity. The melting curves at the two wavelengths of measurements are different for a given complex, because their ultraviolet spectra change differently with temperature. The main observation in these UV melts is that the T_M value for a given complex remains constant over a 10-fold increase in strand concentration, which confirms their intramolecular formation, as shown in Figure 3b. Model-dependent ΔH_{vH} enthalpies, analyzed from the shape of the melting curves, are presented in the first column of Table 1. We obtained an average ΔH_{vH} of 35 ± 5 kcal/mol for the unfolding of the cation complexes with strong CD bands, while the Cs^+–aptamer complex yielded a ΔH_{vH} of 24 ± 5 kcal/mol. This 35 kcal/mol value compares well with the heat of 29–40 kcal/mol for the UV unfolding of one G-quartet stack of three different telomere model sequences[8,23] and is significantly larger than the enthalpy value of 16 kcal/mol enthalpy for the unfolding of two GG/CC base-pair stacks, calculated from nearest neighbor parameters.[24]

The similarities in the CD spectral characteristics and UV melting behavior of the complexes with higher T_M's indicate that Sr^{2+}, Ba^{2+}, K^+, Rb^+, and NH_4^+ induce the aptamers to fold into relative stable intramolecular G-quadruplexes. The coordination of these cations between two G-quartets correlates with the actual size of their ionic radii: K^+ (1.33 Å), Rb^+ (1.47 Å), NH_4^+ (1.43 Å), Sr^{2+} (1.12 or 1.27 Å for a coordination number of 8), and Ba^{2+} (1.34 Å).[25] This correlation indicates that an ionic radius in the range 1.3–1.5 Å is the optimum size for a cation to be sandwiched in the complex; other cations are either too small or too big.[26]

We have further investigated the unfolding of the stable cation–aptamer complexes using calorimetry. The corresponding DSC melting profiles are shown in Figure 4. These curves indicate that all cation–aptamer complexes unfold essentially in monophasic transitions with the exception of the complexes with Sr^{2+} and Ba^{2+} that showed an additional small peak at

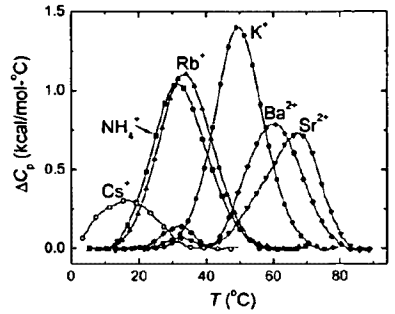

Figure 4. Differential scanning calorimetric curves for the unfolding of the cation–aptamer complexes. We used the following cations: Cs^+, NH_4^+, Rb^+, K^+, Ba^{2+}, and Sr^{2+} under similar solution conditions as indicated in Figure 2.

lower temperatures. The T_M values obtained from the peaks of these curves follow the same trend as that observed in the UV melts: $Sr^{2+} > Ba^{2+} > K^+ \gg Rb^+ \sim NH_4^+ \gg Cs^+$. These T_M values are also included in the plots of Figure 3b and further confirm their intramolecular formation. The unfolding of complexes yielded endothermic heats in the following order: K^+ (25.6 kcal/mol) > $Rb^+ \sim NH_4^+ > Ba^{2+} \sim Sr^{2+}$ (17.3 kcal/mol) $\gg Cs^+$ (6.5 kcal/mol), as shown in Table 1. The calorimetric unfolding heat for the K^+–aptamer complex is in good agreement with the value of 22 kcal/mol reported previously.[11] Integration of the small peaks in the DSC curves of the Ba^{2+}–aptamer and Sr^{2+}–aptamer complexes yielded heats of 1.5 and 0.6 kcal/mol, respectively; these heats are small and may well correspond to unstacking contributions of the TGT loops. Additional peaks are not observed in the K^+, Rb^+, and NH_4^+ thermograms, because these complexes melt at lower temperatures and the unstacking contributions of the TGT loops may be already included in their main transitions. ΔH_{vH} values obtained from analysis of the shape of the DSC curves are shown in the third column of Table 1. The average ΔH_{vH} for these six cations is 33 ± 3 kcal/mol, in excellent agreement with the values obtained in the optical melts. However, the ΔH_{vH} values are much higher than the model-independent heats (ΔH_{cal}), as shown in Table 1. This result suggests that the unfolding of each complex is highly cooperative and consistent with the small dimensions of a cation complex with two G-quartets. An alternate and the least likely explanation for the observed $\Delta H_{vH}/\Delta H_{cal}$ ratios of 1.4 (K^+) and 2.0 (Sr^{2+}) is the formation of aggregate states of two complex molecules, which may be consistent with the aggregation tendency of guanine residues. The Cs^+–aptamer complex also unfolds with a high $\Delta H_{vH}/\Delta H_{cal}$ ratio of 4.5, indicating that the initial state of this complex at low temperatures is a higher aggregated state.

Isothermal Binding Heats for the Interaction of Cation. Isothermal titration calorimetry is used to measure the heats of complex formation at 10 °C and 20 °C. In general, the binding of a cation to the aptamer in the Cs^+ form is accompanied by exothermic heats. The enthalpies at 20 °C range from −24.5 kcal/mol (K^+) to −16.7 kcal/mol (Sr^{2+}), while much lower exothermic heats are obtained at 10 °C, as shown in Table 1. Their magnitude is too large to invoke solely electrostatic interactions, which are normally considered negligible;[27] therefore, other endothermic and exothermic contributions need to be taken into account. These endothermic contributions include

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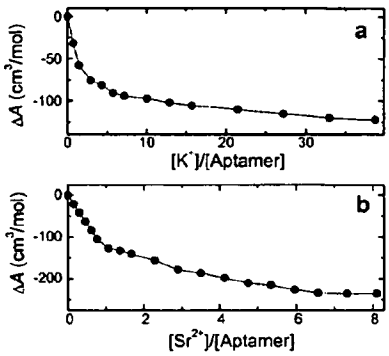

Figure 5. Ultrasonic titration curves of d(GGTTGGTGTGGTTGG) with K^+ (a) and Sr^{2+} (b). All experiments done in 10 mM Cs−Hepes buffer at pH 7.5 and 20 °C. Please note that the scales of the *x*-axes are different.

disruption of stacking interactions of the Cs^+−aptamer complex, removal of electrostricted water molecules from the cation, and the immobilization of structural water by the complex. The exothermic contributions include base stacking interactions in the formation of G-quartets upon cation binding and the removal of structural water from the existent fraction of random coils at the particular measuring temperature. The lower heats obtained in the titrations at 10 °C indicate that a higher fraction of Cs^+−aptamer complex forms at this temperature; this is consistent with its unfolding transition that takes place at lower temperatures. At the same time, a higher fraction of cation−aptamer complex forms at 10 °C, as in the case of the Rb^+ and NH_4^+ complexes. In the titration experiments at 20 °C, the formation of the K^+−aptamer, Ba^{2+}−aptamer, and Sr^{2+}−aptamer complexes is almost ideal, because these complexes formed completely and the Cs^+−aptamer complex is ~70% disrupted. The disruption of the remaining 30% of the Cs^+ complex amounts to an endothermic contribution of ~2 kcal/mol. Overall, good agreement is obtained between the isothermal heats at 20 °C and the unfolding heats, as shown in Table 1, indicating negligible heat capacity effects. Furthermore, the titration experiments show that K^+ or Sr^{2+} binding to the aptamer, or vice versa, yields similar exothermic heats. This indicates that these processes are in truly physical and chemical equilibrium and that aggregation of complexes can be ruled out. The average heat of these titrations yielded enthalpies of −25.7 kcal/mol and −16.8 kcal/mol for the K^+ and the Sr^{2+} complexes, respectively, as shown in Table 1. Their large difference of −8.9 kcal/mol (8.3 kcal/mol if we use the calorimetric unfolding heats) suggests that K^+ and Sr^{2+} have different hydration contributions. These contributions may arise from differences in both their hydration states[28,29] and the actual release of electrostricted water upon binding to the aptamer to form the complex.

Hydration Parameters. We have used density and ultrasonic techniques to determine the changes in the molar volume and molar adiabatic compressibility for the folding of complexes. The change of the concentration increment of ultrasonic velocity, ΔA, of a Cs^+−aptamer solution is measured during the course of a titration with K^+ or Sr^{2+}. The resulting curves are shown in Figure 5. These acoustic titration curves reveal that an increase in cation concentration is accompanied by a decrease in the increment of ultrasound velocity. The curves show an initial sharp decrease of ΔA, which corresponds to complex formation, followed by a gradual leveling off. In the K^+ curve, the initial decrease takes place up to a $[K^+]$/[aptamer] ratio of 3, while in the Sr^{2+} curve the decrease occurs up to a $[Sr^{2+}]$/[aptamer] ratio of 1. This indicates the formation of tighter complexes with Sr^{2+} because of its higher charge. After these ratios, the overall changes in ΔA are small for K^+ and very large for Sr^{2+} and may correspond to the hydration effects for the interaction of cations with the surface of the cation−aptamer complexes.[22,30]

Table 2. Complete Thermodynamic Profiles for the Formation of G-Quaduplexes at 20 °C[a]

cation	T_M[b]	$\Delta G°$[c]	ΔH_{cal}[c]	$T\Delta S$[c]	ΔH_{ITC}[c]	ΔV[d]	$\Delta K_S \times 10^4$[e]
K^+	49.1	−2.3	−25.6	−23.3	−24.5 (−26.8)	−15.9	98.5
Sr^{2+}	66.9	−2.4	−17.3	−14.9	−16.7 (−16.9)	−15.3	108.4

[a] All values determined in 10 mM Cs−Hepes buffer containing 50 mM KCl or 10 mM $SrCl_2$, at pH 7.5. The T_M (±0.5 °C), $\Delta G°$ (±5%), ΔH_{cal} (±5%), and $T\Delta S$ (±7%) were determined in DSC experiments. The ΔH_{ITC} (±4%) values were determined in ITC experiments, while ΔV (±15%) and $\Delta\Phi K_S$ (±10%) were determined in density and ultrasonic measurements. The ITC enthalpy values in parentheses correspond to reverse titrations. [b] Units of °C. [c] Units of kcal/mol. [d] Units of cm³/mol. [e] Units of cm³/mol·bar.

The ΔA values obtained at [cation]/[aptamer] ratios of 1 (Sr^{2+}) and 3 (K^+), together with parallel density measurements, were used to characterize the hydration parameters that take place in the formation of the cation−aptamer complexes at 20 °C. The volume and compressibility effects for the formation of G-quadruplexes with K^+ and Sr^{2+} are shown in the last two columns of Table 2. We obtained negative values for the volume change, indicating a decrease in the total volume of the system. This shows that complex formation with K^+ and Sr^{2+} is accompanied by an uptake of water molecules, because the molar volume of water around a solute is considered lower than that of bulk water.[31] On the other hand, the changes in the molar compressibility parameter are negative, which indicate the opposite, a release of water molecules. This apparent discrepancy on the opposite signs of these two parameters invokes the participation of two types of water molecules, electrostricted (around charged atomic groups) and hydrophobic or structural (around polar and nonpolar groups), on the formation of cation−aptamer complexes. Therefore, the overall hydration effects accompanying the binding of each cation correspond to a partial conversion of electrostricted water to hydrophobic water. However, a closer look on the physical events that take place on complex formation indicates that these hydration parameters are the result of mainly two contributions: the uptake of water due to the conformational reorganization of forming a folded G-quadruplex from the random coil state (or some other form in Cs^+) and the cation dehydration upon binding tightly to the quadruplex core. The hydration contribution due to the atmospheric binding of counterions is considered negligible at these particular [cation]/[aptamer ratios].[22] In the particular case of Sr^{2+}, its hydration contribution can be estimated from similar parameters of its binding to EDTA where the cation loses ~80% of its hydrating water.[32] The volume and compressibility effects for the formation of a Sr^{2+}−EDTA complex are 40.5 cm³/mol and 116 × 10^{-4} cm³/mol·bar, respectively,[32] and correspond

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to a release of electrostricted water, because its $\Delta V/\Delta K_S$ ratio is equal to 0.35×10^4.[29,33,34] To estimate the hydration contribution for the folding of the oligonucleotide into the Sr^{2+}–aptamer complex, we have adjusted the above parameters to take into account the coordination number of Sr^{2+} in this complex. This estimation yields a volume effect of -75 cm^3/mol and a compressibility effect of -62×10^{-4} cm^3/mol·bar; the negative signs of these parameters show an overall uptake of water molecules. The resulting empirical $\Delta V/\Delta K_S$ ratio[34] of 1.2×10^4 bar suggests an uptake of hydrophobic water. This water may well be immobilized on the surface of the complex and around the constrained loops.

Thermodynamic Profiles for the Formation of G-quadruplexes with K^+ and Sr^{2+}. Complete thermodynamic profiles for the formation of the K^+–aptamer and Sr^{2+}–aptamer complexes at a comparable ionic strength and 20 °C are presented in Table 2. The favorable $\Delta G°$ terms are similar in magnitude and result from the characteristic compensation of a favorable enthalpy term with an unfavorable entropy term. The favorable enthalpy term is the result of an exothermic heat from the stacking of two G-quartets that overrides the endothermic contribution of the release of electrostricted water from the cation. The endothermic contributions of disrupting base–base stacking interactions of the single strands are considered negligible, because the unfolding heats are similar in magnitude to the isothermal heats for both cation–aptamer complexes. The unfavorable entropy term results from contributions of the ordering of both a single strand and cation upon folding into a G-quadruplex and the overall immobilization of hydrophobic water by the complex. However, the magnitude of the enthalpy–entropy compensation is larger for the K^+–aptamer complex; the enthalpy term is more favorable (by -8.9 kcal/mol) despite its lower T_M (by ~18 °C). This enthalpy difference may be explained in terms of several contributions: the hydration state of the free cations, yielding a differential release of water upon binding to the oligonucleotide; differential hydration of the cation–aptamer complexes; and different base-stacking contributions of the complexes.

To estimate these hydration contributions, we need to consider the dehydration effects of cations and O atoms upon coordination of the cation to eight O6 atoms of guanine and the hydration effects of quadruplex formation. The dehydration effects of each cation and O atoms are estimated using the molar compressibility values of 28×10^{-4} cm^3/mol·bar (K^+),[28,29] 75×10^{-4} cm^3/mol·bar (Sr^{2+}),[29] and 10×10^{-4} cm^3/mol·bar (O),[35] and the change in adiabatic compressibility of 8.1×10^{-4} cm^3/mol·bar for the transfer of water from the hydration shell of the ion to the bulk state.[33] These estimations yield overall dehydration effects of ~3 electrostricted water molecules for K^+, ~9 for Sr^{2+}, and 1 water molecule per O atom. Because the hydration contribution of the single strand (in the Cs^+ form) is similar for each reaction, Sr^{2+} would yield a higher release of six electrostricted water molecules. The hydration contribution for the rearrangement of a single-strand into a G-quadruplex is estimated from the experimental compressibility terms of Table 2. These compressibility values were corrected by the compressibility of the dehydration of cations and oxygen groups of guanine to yield -9.5×10^{-4} cm^3/mol·bar (K^+ complex) and -46.6×10^{-4} cm^3/mol·bar (Sr^{2+} complex). The uptake of hydrophobic water by each complex is estimated by dividing these values by the compressibility of water when immobilized around polar and nonpolar groups, assumed to be equal to -3.2×10^{-4} cm^3/mol·bar.[36] This exercise yields an immobilization of ~3 and ~15 hydrophobic water molecules around the K^+ and Sr^{2+} complexes, respectively, reflecting differences in the actual structure of complexes that resulted in different exposures of polar and nonpolar groups.

In terms of enthalpy contributions and relative to the formation of the K^+–aptamer complex, the differential dehydration effect of Sr^{2+} ions (6 electrostricted waters) corresponds to an endothermic heat contribution of ~1.8 kcal;[37] therefore, the remaining differential heat of 7.1 kcal/mol is due to differences in the stacking of G-quartets and/or endothermic contributions of the differential immobilization of 12 hydrophobic waters on the surface of the Sr^{2+} complex. We speculate that, depending on the extent of stacking interactions, the additional uptake of hydrophobic waters by the Sr^{2+} complex contributes to an endothermic heat ranging from 0 kcal/mol to 0.6 kcal/mol.

Concluding Remarks

We initially used CD spectroscopy and UV melting techniques to determine the conformation and unfolding thermodynamics of G-quadruplexes with a variety of monovalent and divalent metal ions. The CD spectra and melting profiles as a function of strand concentration showed that K^+, Rb^+, NH_4^+, Sr^{2+}, and Ba^{2+} are able to form stable intramolecular cation–aptamer complexes at temperatures above 25 °C. The cations Li^+, Na^+, Cs^+, Mg^{2+}, and Ca^{2+} form weaker complexes at very low temperatures. These results have been rationalized in terms of their ionic radii; cations with an ionic radius in the range 1.3–1.5 Å fit well within the two G-quartets of the complex, while the other cations cannot. DSC and ITC techniques were used to characterize the unfolding and folding of cation–aptamer complexes with K^+, Rb^+, NH_4^+, Sr^{2+}, and Ba^{2+}. The heat for the unfolding of each complex is in excellent agreement with the folding heat of isothermal titration experiments, indicating negligible contributions from base stacking interactions of the single strands. We have further characterized the hydration contributions for the formation of G-quadruplexes with K^+ and Sr^{2+} using density and acoustical techniques. The overall thermodynamics parameters showed that the Sr^{2+}–aptamer complex unfolds with a higher transition temperature and lower endothermic heat, but its favorable formation (in terms of $\Delta G°$) is comparable to that of the K^+–aptamer complex. The overall hydration effects of complex formation yielded two main contributions, dehydration of both cations and guanine O6 atomic groups and the water uptake upon folding of a single strand into a G-quadruplex structure.

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EXHIBIT G

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Nucleic Acid Aptamers—From Selection in Vitro to Applications in Vivo

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ABSTRACT

Aptamers are nucleic acid ligands which are isolated from combinatorial oligonucleotide libraries by in vitro selection. They exhibit highly complex and sophisticated molecular recognition properties and are capable of binding tightly and specifically to targets ranging from small molecules to complex multimeric structures. Besides their promising application as molecular sensors, many aptamers targeted against proteins are also able to interfere with the proteins' biological function. Recently developed techniques facilitate the intracellular application of aptamers and their use as in vivo modulators of cellular physiology. Using these approaches, one can quickly obtain highly specific research reagents that act on defined intracellular targets in the context of the living cell.

The synthesis and functional screening of large libraries of compounds is commonly known as "combinatorial chemistry". In recent years, a number of methods have been developed to isolate molecules with desired functions from libraries of small organic molecules, nucleic acids, proteins, peptides, antibodies or single-chain antibody fragments (scFv), or other polymers (for comprehensive reviews on these topics, see ref 1). The identification of active compounds from composite mixtures proceeds in iterative cycles of selection and amplification. This not only permits screening of libraries with high complexity but also facilitates further increases in the library diversity by mutating the pool during amplification steps. These types of selection consequently require encoding strategies that allow unambiguous resolution of the composition and sequence of any active molecules, information required for their subsequent amplification or optimization. With peptide, protein, or antibody fragment libraries, this is most easily and logically achieved by approaches such as displaying peptides, proteins, or antibody fragments on phage and cell surfaces, which convert the amino acid sequence into genetic information that can be amplified in vivo. Alternatively, two encoding strategies have been developed recently that facilitate entirely in vitro synthesis and selection of proteins: mRNA-protein fusions[2] and ribosome display.[3]

Nucleic acids are particularly suited for combinatorial selection approaches because they can fold into well-defined secondary, tertiary, and quaternary structures and they are easily amplified by the polymerase chain reaction (PCR) or in vitro transcription. By their very nature, nucleic acids provide the blueprint for their own replication and, by that same token, for their improvement and optimization. Consequently, the sequence space that can be successfully screened in parallel is the most extensive among all the combinatorial chemistry techniques: far more than 10^{15} different molecules can be screened simultaneously for a particular function. In vitro selection of combinatorial oligonucleotide libraries can lead to the isolation of nucleic acids such as RNA, ssDNA, modified RNA, or modified ssDNA that bind a wide variety of targets with high specificity and affinity[4,5] (for a recent review, see ref 6). But it is another feature that makes them enormously flexible and powerful: possessing highly selective molecular recognition properties, nucleic acids can target key molecules inside or outside a diseased cell, or may be used like antibodies for diagnostic purposes. Therefore, we have chosen this unique class of "dual mode" molecules, called aptamers, to form the basis of a simple yet highly versatile molecular toolbox.

Isolation and Application of Aptamers Binding Small Molecules

Aptamer structures have been comprehensively reviewed and discussed in a number of commentaries and reviews.[7−9] Comparisons of various ligand-binding aptamer structures with proteins that bind related molecules have shown that nucleic acids and proteins use strikingly similar strategies for the formation of well-defined binding pockets.[10] Structural studies performed with aptamer/ligand complexes have revealed insights into principles of folding, shape, and surfaces, as well as the molecular diversity associated with nucleic acid architecture, molecular recognition, and adaptive binding.[11]

Our first steps into combinatorial nucleic acid selection led to the isolation of aptamers which bind to small molecules such as amino acids and biological cofactors[12−16] (for a recent review, see ref 17; the first aptamer selected for a biological cofactor was an ATP-binding RNA sequence,[18] the solution structure of which was also elucidated by NMR spectroscopy[19−21]). Like others in this field,

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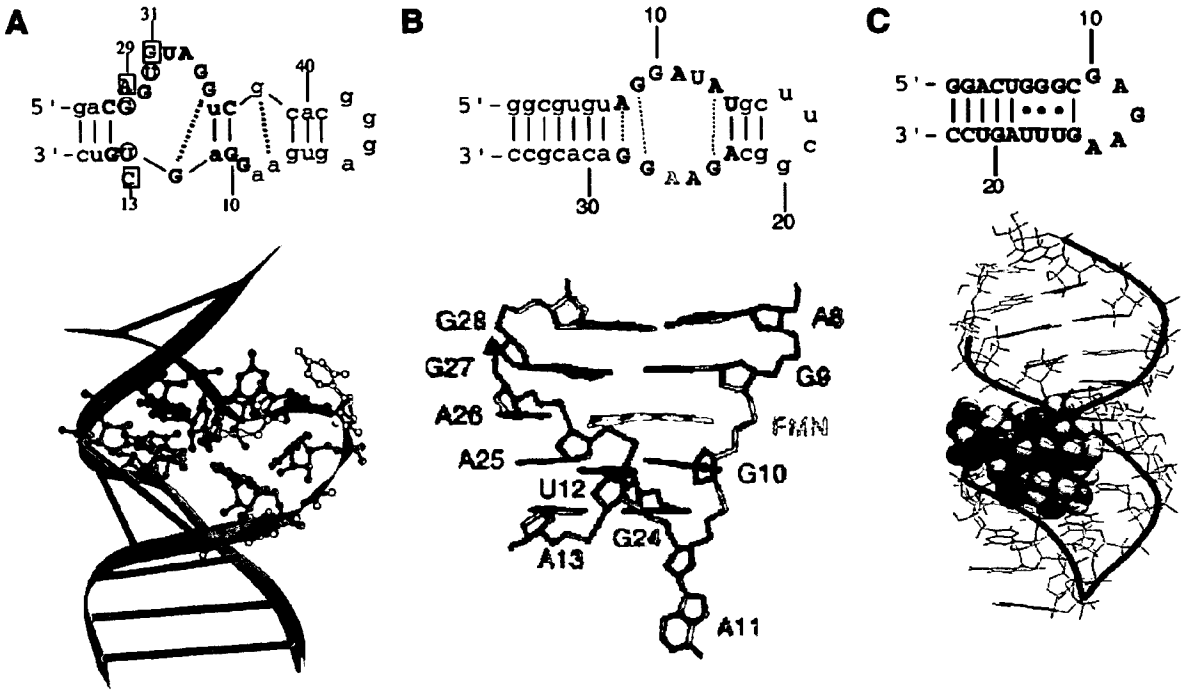

FIGURE 1. Structures of some aptamers from our group. (A) Secondary structure proposed previously for the citrulline- and arginine-specific aptamers, based on covariations of selected sequences and on the chemical footprinting pattern obtained in the presence of the cognate amino acid, as well as in damage selection experiments. The bases which were conserved among different isolates are shown in uppercase, while variant bases are in lowercase. The three nucleotides critical for amino acid specificity (13, 29, and 31) are indicated by circles (for citrulline) and boxes (for arginine). The tertiary structure is shown for the citrulline-binding aptamer.[23] (B) Secondary structure of the FMN aptamer and tertiary structure of the FMN-binding region in this aptamer as determined by NMR spectroscopy.[30] (C) Secondary structure of the neomycin B aptamer[15,27] and tertiary structure of this aptamer as determined by NMR spectroscopy.[29]

we wanted to investigate certain principles of molecularevolution and recognition of specific ligand-binding RNA molecules. For example, an RNA aptamer specifically recognizing the amino acid L-arginine was "evolved" from an in vitro-selected L-citrulline-binding parent sequence.[12] The two aptamers differ by only three mutations, yet each exhibits very high specificity for its cognate ligand. The three-dimensional fold defined by chemical probing analysis and NMR spectroscopy showed how the three mutations within the amino acid binding site of these RNAs determine which of the two amino acids is specifically recognized (Figure 1A).[22,23]

The structures of the citrulline and arginine aptamers, together with those of several other arginine aptamers for which structural data exist, were used as an experimental starting point to analyze a hypothesis that the genetic code might have evolved via mechanisms of molecular recognition between amino acids and short RNA motifs.[24] Statistical evidence suggests that arginine aptamers, together with several other arginine aptamers for which structural data exist, appear to have a significant bias in favor of arginine codons at their binding sites.[25] These data support the hypothesis that amino acids can specifically interact with RNA sequences that contain their cognate codons. In the meantime, similar evidence has also been obtained for other aptamer/amino acid complexes (M. Yarus, personal communication).

The degree of molecular discrimination achieved by aptamer/small molecule complexes can match or even surpass that of antibodies. An aptamer specific for theophyllin distinguishes it from caffeine, which differs by only one methyl group, at least 10-fold more efficiently than an antibody isolated for the same purpose.[26] An aptamer selected for specific binding to L-arginine shows a 12 000-fold reduced affinity to the D-arginine enantiomer.[14]

Aptamers for small molecules,[17] such as neomycin[15,27] and FMN,[13] have been used in surface plasmon resonance technology to generate target-specific biosensors.[28] The three-dimensional structures of the FMN and neomycin B aptamers[29] have also been solved by Patel and colleagues[30] (Figure 1B,C). This and other analyses[31,32] have revealed insights into the mode and dynamics of molecular recognition of a dimethylisoalloxazine moiety by an RNA aptamer.

One of the remarkable characteristics of in vitro-selected RNA aptamers is that ligand binding is always accompanied by significant structural changes in the binding RNA molecule.[11] Ligands seem to become an integral part of the RNA aptamer structure once they are bound.[8,33] This property of aptamers might have inspired

Table 1. Selected Targets for Aptamers That Recognize Extracellular Proteins

target	K_d (nM)	nucleic acid	ref
acetylcholine receptor	2.0	RNA	70
L-Selectin	3.0	RNA	71
basic fibroblast growth factor (bFGF)	0.35	RNA	72
platelet-derived growth factor (PDGF)	0.1	ssDNA	73
keratinocyte growth factor (KGF)	0.0003	2′-modified RNA	43
vascular endothelial growth factor (VEGF)	0.14	2′-modified RNA	44
interferon-γ (IFN-γ)	6.8	2′-modified RNA	74
cellular prion protein (PrP^c)	nd	RNA	40
anti-acetylcholine autoantibodies	60	2′-amino-RNA	42

the idea of fusing aptamer sequences with known catalytic RNAs, incorporating the principle of allosteric regulation into ribozyme catalysis. None of the existing natural ribozymes were known to operate as allosteric enzymes in vitro or in vivo. Breaker and his colleagues[34,35] and Araki et al.[36] integrated sequences of the ATP,[18] theophylline,[26] or FMN aptamers[13] into the hammerhead ribozyme (HHR) to rationally engineer allosteric HHRs, which carry out the phosphodiester cleavage—or inhibit it—only after the relevant ligands have been added to the cleavage buffer.[34,35] In the case of the FMN aptamer, modular rational design and in vitro selection techniques were combined to generate precision molecular switches comprising ribozyme/aptamer chimeras.[35] Intracellular ribozymes which target and inactivate certain mRNAs are currently under investigation for their use to control the expression of proteins. Allosteric ribozymes which are activated or inhibited by membrane-permeable, nontoxic, low-molecular-weight molecules may provide extremely powerful tools for conditional gene expression.[37] It is now possible to directly select for aptameric regulatory RNA motifs that activate a "silenced" ribozyme[38] or that inhibit an active ribozyme (Piganeau, N.; Jenne, A.; Thuillier, V.; Famulok, M., manuscript submitted) in the presence of small organic molecules. A more direct but nevertheless efficient route to use aptamers inside cells for the control of protein expression was recently described.[39] Placing an aptamer specific for the organic dye Hoechst 33528 in the 5′-untranslated region (5′-UTR) of a reporter gene mRNA prevented its translation in the presence of the cognate ligand, whereas gene expression proceeded normally in its absence.

Functional Aptamers for Proteins and Their Application in Biotechnology, Molecular Medicine, and Diagnostics

The specificity of molecular recognition combined with the ease by which protein-binding aptamers can be isolated, engineered, evolved, and modified chemically—exclusively ex vivo—makes these molecules very attractive as tools in molecular medicine, biotechnology, and diagnostics. Consequently, the vast majority of aptamers that have been isolated so far are for specific binding to protein targets. Table 1 summarizes some examples of aptamers that recognize proteins that are expressed on cell surfaces or are localized extracellularly. Among them are aptamers composed of ssDNA, RNA, or chemically modified nucleic acids, where a repertoire of chemically introduced functional groups increases oligonucleotide stability and functional group diversity.

We have recently applied in vitro selection to isolate RNA aptamers that are directed against the Syrian golden hamster cellular prion protein PrP23-231 (PrP^c). A recombinant PrP/glutathion-S-transferase (GST) fusion protein immobilized on glutathion agarose was used for the selection.[40] Sequence comparisons suggested that all aptamers isolated are likely to contain a three-layered G-quartet as a structural element critical for PrP recognition. Mapping experiments with GST-PrP peptides revealed that the region of PrP^c critical for aptamer binding spanned the N-terminal amino acids 23−52. Individual radiolabeled aptamers specifically recognized authentic prion protein in brain homogenates from various species, such as wild-type mice, hamster, and cattle, as demonstrated by supershifts obtained in the presence of PrP-specific antibodies, but no interaction was observed with brain homogenates from PrP knock-out mice. This study showed that aptamers are able to recognize their specific target among the hundreds of different proteins present in tissue homogenates.

Nuclease-Resistant Functional Aptamers

The PrP-binding aptamers probably possessed sufficient stability in crude brain homogenates due to the stable G-quartet scaffold protecting them from exonuclease degradation. Small protein-binding RNA aptamers, however, usually do not contain stabilizing structural scaffolds of this kind. Therefore, for them to be widely applicable as potential therapeutics, diagnostics, or assay components, the capacity of aptamers to evade nuclease degradation has to be increased. A number of studies have established that functional oligonucleotides can be made to be not only strikingly small but also resistant to degradation in biological materials.[41] One possible way to circumvent the vulnerability of RNA to nuclease degradation is indirect: in a first step, an aptamer that binds the enantiomer of the target is selected, then, in a second step, the enantiomer of the aptamer is synthesized (from L-phosphoramidites) as a nuclease-insensitive ligand of the natural target. This mirror-image approach has been applied to L-arginine, D-adenosine, and the peptide hormone vasopressin (for review, see refs 6 and 41).

An alternative approach is the direct selection of an aptamer from libraries of modified RNAs. Modifications must be chosen that are compatible with nucleic acid replicating enzymes such as reverse transcriptase or DNA and RNA polymerases. The modifications most commonly used are those where the 2′-OH group of pyrimidines is substituted by a 2′-fluoro or 2′-amino group. 2′-Amino-modified nuclease-resistant aptamers have been selected which bind to autoantibodies of patients affected by the muscular disease *myasthenia gravis*. Such aptamers inhibit

the binding of these autoantibodies to acetylcholine receptors on human cells, blocking the associated pathogenic consequences.[42] Similarly, 2′-fluoro-modified nuclease-resistant aptamers directed against the human keratinocyte growth factor block its activity with a K_i of 34 pM.[43]

An aptamer with 2′-aminopyrimidine modifications selected for binding to vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) was minimized to a modified 24-mer. The 2′-OH groups of defined purine residues were subsequently modified by 2′-methoxy groups in a damage selection experiment where variants of this aptamer chemically synthesized with a mixture of 2′-OH- and 2′-OCH_3-purines were screened for enhanced binding to VPF/VEGF.[44] 2′-Methoxy-substituted purine residues in this aptamer were identified by protection from alkaline hydrolysis. Of the 13 purines in the 24-mer, nine could be substituted by 2′-methoxy purine, while the other four could not be changed without significant loss of binding affinity. The end result was a modified aptamer which bound to the target protein with a K_d of 0.14 nM and specifically blocked the binding of ^{123}I-labeled VPF/VEGF to cell surface receptors expressed on human umbilical vein endothelial cells.

These and many other examples show that aptamers can routinely be obtained against almost any desired target. Most of the targets summarized in Table 1 are either located on the cell surface or otherwise easily accessible to a nucleic acid aptamer. Because most aptamers are also capable of very specifically modulating the biological function of their target, these molecules are potentially excellent candidates for drugs or drug leads.

Functional Aptamers in Vivo

Recently, our interest turned to using aptamers for another fascinating purpose. As RNA molecules, aptamers can be synthesized directly by the cell's own transcription machinery. By being expressed within the cell, aptamers can be used to target a certain protein in its natural environment. Due to their high affinities and specificities, such intracellular aptamers (for which the term "intramers" was coined [45]) may provide excellent tools to specifically inhibit signal transduction, cell growth, transcription, translation, or other intracellular processes or to study RNA−protein interactions in vivo.

Intramers in Procaryotes and in the Nucleus

Several studies already existed where RNA aptamers isolated in vitro were expressed in vivo to study their biological function within cells. In most cases the question was whether aptamers are capable of functionally replacing a natural RNA sequence in vivo. Among those tested were anti-HIV-1 Rev aptamers, which were inserted into the full-length Rev-responsive element (RRE) in place of the Rev-binding element (RBE). They were found to be functionally equivalent to the wild-type RBE in their ability to mediate Rev function in vivo.[46] Our laboratory took a different initial approach to investigate in vivo-expressed aptamers in procaryotes. We had selected a series of aptamers that bind the special elongation factor SelB[47,48] (for a commentary, see ref 49) In *Escherichia coli*, SelB is required for the co-translational incorporation of the unusual amino acid selenocysteine into proteins such as the formiate dehydrogenases. To do this, SelB binds simultaneously to selenocysteyl-tRNA^{Sec} and to an RNA hairpin structure located directly 3′ of the selenocysteine opal (UGA) codon in the mRNA of formiate dehydrogenases (*fdhF*) (Figure 2).

Using a doped pool of this mRNA hairpin, we were able to select a mutated version of this motif which also had the ability to bind tightly and specifically to SelB. To dissect SelB binding to the *fdhF* mRNA hairpin from the overall biological activity of this complex, four selected aptamers were analyzed in vivo for UGA readthrough in a *lacZ* fusion construct. Of these, only one promoted UGA readthrough in vivo. The other three aptamers, despite their secondary structures and binding affinities being similar to those of the wild-type motif, were severely impaired or unable to replace the *fdhF* mRNA hairpin function in vivo. This finding implies that the functions of the *fdhF* hairpin go beyond the mere tethering of selenocysteyl-tRNA^{Sec} to the UGA codon via SelB.

RNA aptamers against yeast polymerase II were selected in a collaborative study with the laboratory of A. Sentenac, Gif-sur-Yvette, France. These oligonucleotides revealed specificity for polymerase II but do not inhibit polymerase I or polymerase III. The aptamers were found to interact preferentially with the two largest subunits of pol II, B220 and then B150. In vivo inhibition studies showed that, in yeast cells with an artificially reduced level of endogenous polymerase II, pol III-dependent expression of binding aptamers caused a cell growth defect, whereas expressing control RNAs did not[50] (Figure 3).

Shi et al. selected RNA aptamers that bind the RNA-binding protein B52.[51] B52 is a member of the SR protein family,[52] a class of nuclear proteins that play an essential role during pre-mRNA splicing in *Drosophila melanogaster*. To elucidate the function of selected RNA aptamers in vivo, a pentameric aptamer was constructed and expressed in transgenic flies under the control of an inducible promotor. It was demonstrated that B52 colocalizes with the pentameric aptamer at its sites of insertion in the polytene chromosome, indicating that the aptamer interacts with B52 in vivo. Since the level of B52 is critical for *Drosophila* development, deletion or overexpression of B52 leads to enhanced lethality and morphological defects. Shi et al. demonstrated that induced expression of the RNA aptamer in transgenic flies resulted in 50% higher lethality, presumably due to a reduced amount of available B52, the rest being sequestered by bound RNA aptamer. A second transgenic fly model that can overexpress both B52 and the RNA aptamer was used to further characterize the effects of the aptamer in vivo. In this model the authors showed that all effects caused by B52 overexpression could be reversed by induced expression of the RNA aptamer.

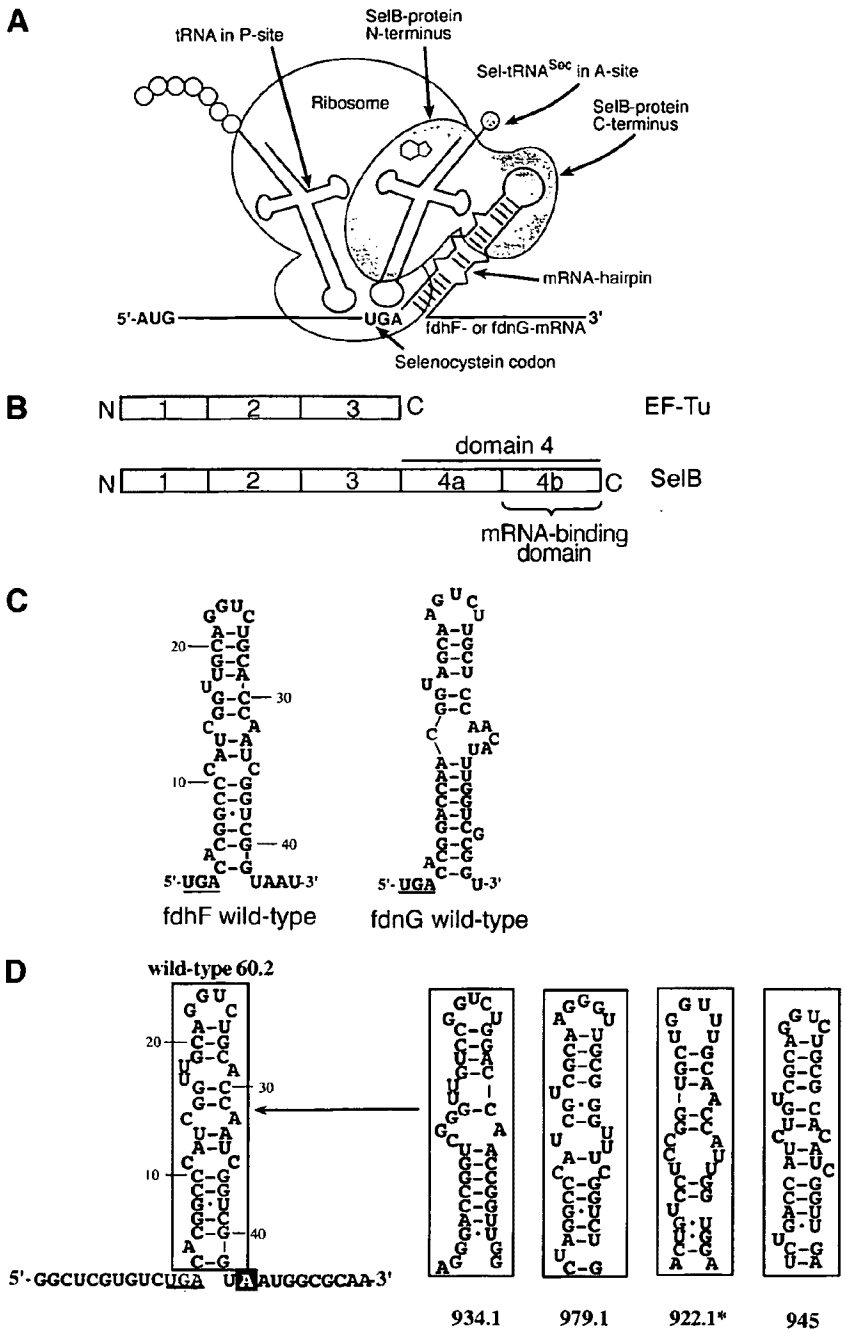

FIGURE 2. SelB protein and the *fdhF* and *fdnG* mRNA hairpins. (A) Schematic representation of the processes occurring during the cotranslational incorporation of selenocysteine at the ribosome. The quaternary complex between SelB, GTP (hexagon), selenocysteyl-tRNA^{Sec}, and the *fdhF* or *fdnG* mRNA hairpin is attached to the ribosome. (B) Schematic representation of SelB and its derivatives. Bottom: the full-length SelB protein (aa 1−614). The protein domain 4b corresponds to the ultimate C-terminus of SelB (aa 472−614). In the truncated version SelB1−474, the C-terminal domain 4b was deleted. This derivative shows extensive homologies to EF-Tu (top). (C) Secondary structures of the *fdhF* and *fdnG* mRNA hairpin motifs located immediately 3′ of the UGA selenocysteine codon. (D) Constructs for testing in vivo activity of SelB-binding aptamers. Aptamer sequences shown in the boxes (right) were substituted for the boxed region in the wild-type sequence (left). These constructs were used in a UGA stop codon readthrough assay using *lacZ* as the reporter gene.[47]

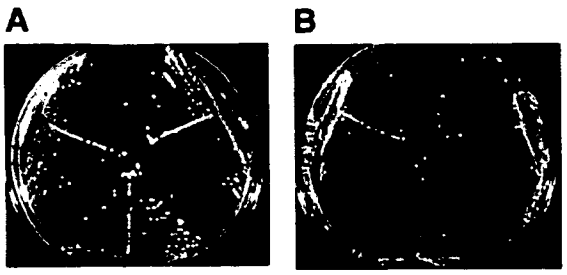

FIGURE 3. Growth of yeast strain YF1971 cells expressing Pol II-specific aptamer RNA (B) compared to nonbinding RNAs from the unselected pool (A). Growing these cells in a leucine-rich medium leads to a reduction in the endogenous amount of Pol II. Aptamer-expressing cells clearly showed reduced growth under conditions of reduced Pol II expression.

Intramers as Tools in Functional Genomics

So far, successful in vivo expression of aptamers had been restricted to target proteins with a natural affinity for nucleic acids (Rev, SelB, Pol II, B52) and, in the case of eukaryotes, to a limited cellular compartment, the nucleus. We therefore decided to devote further research efforts to the question of whether techniques for in vivo expression of aptamers could be expanded to cover a much broader scope, i.e., to target proteins with no intrinsic affinity for nucleic acids and to include other cellular compartments. As a start in this direction, we were interested in ablating the protein function of cytoplasmic targets with intacellular aptamers or intramers, since we believe this technology could have considerable impact on disease treatment and functional genomics. The emerging field of functional genomics is dedicated to unmasking the physiological role of a gene product, particularly within the dynamic context of the interactive network of a cell where any information received from the environment is processed and results in a cellular response. In other words, functional genomics is an approach which links gene sequences obtained by various genome sequencing projects[53-57] to the function of the proteins they encode, including a huge number of gene products about which nothing is known.[58,59]

Similar to intracellular antibodies (intrabodies[60-62]), intracellularly expressed chemokines (intrakines), or peptide aptamers,[63,64] intramers promise to be an important tool in the attempts to inactivate a gene product without altering the genetic material. Like other intracellular modulators of protein function, intramers provide a conceptually simple approach to phenotypically knocking out protein function without altering the actual proteome status of a cell. Modulating a protein target within the context of its actual expression status inside a living cell would allow simpler and more focused analyses of its biological function and of its value as a potential pharmaceutical target. In contrast to genetic knock-out strategies, these molecules can be used to recognize different protein domains, subdomains, catalytic centers, or post-translational modifications within the same protein, allowing a direct investigation of functional epitopes at the molecular level. Intracellularly expressed modulators of protein function could provide a high-resolution picture of the function of a protein in the context of a living cell. Why is this important?

Most approaches to inhibiting a cellular component rely on investigating phenotypes of a cell or organism that differ from the wild-type as a result of altering the genetic information either by knock-out technologies or by expression/overexpression of a protein or one of its mutant derivatives. As the target protein is probably involved in continuous cross-talk with other cellular components participating in the various regulatory pathways, its absence or overrepresentation is likely to alter the cellular composition, activity, or distribution of other molecules. Despite the power of these techniques, such effects can complicate the interpretation of an observed altered phenotype: it could be due to specifically targeting a key player that regulates the observed phenomenon, or alternatively to unspecific deregulation of the regulatory system responsible. A good example of the complex effects caused by depleting a protein are the studies of the genome-wide transcriptional circuitry in yeast. Knock-out of the cyclin-dependent kinase Srb10, which is implicated in transcriptional regulation, resulted in the derepression of over 170 genes as well as the repression of a few others.[65,66] In addition to the problems associated with such complex effects, these approaches in functional genomics hardly ever distinguish between different domains or posttranslationally modified forms of the same protein. Thus, it is highly desirable to develop and apply methods that permit functional investigations of gene products in the natural context of a cell's proteome. We reasoned that intramers should be perfectly suited to act in various cellular compartments within the context of a living cell and thus help to fulfill the demand for specific intracellular inhibitors or modulators.

Cytoplasmic Intramers

As initial proof that this concept is valid, we recently developed a system that allows the expression of cytoplasmic intramers.[45] As a first target, we chose the cytoplasmic domain of the human $\alpha L\beta 2$ integrin subunit (CD18cyt), a transmembrane protein involved in various regulatory steps in immunology and cell adhesion, found exclusively on leukocytes. The $\beta 2$ integrins are a family of heterodimeric transmembrane proteins whose extracellular domains mediate the adhesion of leukocytes in immune and inflammatory responses by binding to the intercellular adhesion molecule-1 (ICAM-1) expressed on the surface of endothelial cells. The cytoplasmic domains of the integrin α and β chains are thought to be involved in the transmission of signals from inside the cell across the plasma membrane to the surface—a process that is also referred to as "inside-out" signaling.[67,68]

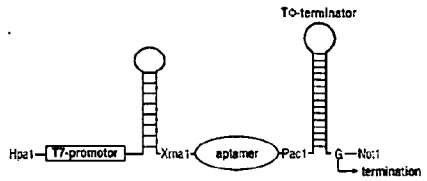

FIGURE 4. Design of the T7 RNA expression cassette. In the TR vector the T7 promoter is located upstream of aptamer-encoding DNA, which is inserted between 5′- and 3′-stem-loop structures. These serve as both RNA-stabilizing motifs and a termination signal for the T7 transcript. Aptamers retain full binding activity when inserted between the stabilizing stem-loops.

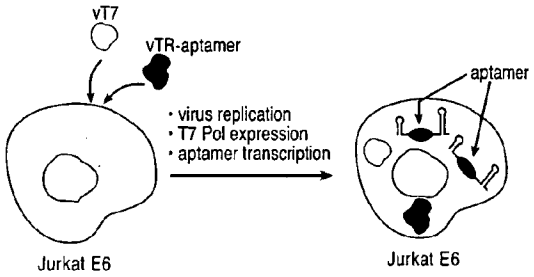

FIGURE 5. Schematic representation of the vaccinia virus-based RNA expression system.

The RNA aptamers used for this approach were isolated by in vitro selection against a synthetic, agarose-immobilized peptide of 46 amino acids that comprised the cytoplasmic domain of the β_2 integrin LFA-1, or CD18. To allow endogenous expression and investigation into their biological function in vivo, aptamer sequences were cloned into an RNA expression cassette, the TR vector. In this vector, the T7 promotor is located upstream of aptamer-encoding DNA, which is in turn inserted between 5′- and 3′-stem-loop structures that serve as RNA-stabilizing motifs and are required for correct termination of the T7 transcripts (Figure 4).

By native gel-shift assays it was shown that aptamers retain full binding activity, even when located between the stabilizing stem-loops. We then infected Jurkat E6 and peripheral blood mononuclear cells (PBMCs) with vaccinia viruses that encode the intramer sequences. Infection with a second vaccinia virus encoding for the T7 RNA polymerase was required for endogenous intramer expression (Figure 5).

Adhesion assays demonstrated that the intramers specific for the cytoplasmic domain of β_2 integrin can block phorbolester-stimulated cell adhesion to immobilized ICAM-1 in Jurkat E6 cells and PBMCs.

This study answered a number of questions that arose before we considered using intramers as modulators of cytoplasmic regulatory pathways; it showed that (i) aptamers can be inserted between different sequence motifs without loss of function, (ii) functional intramers can be expressed at high levels in the cytoplasm, and (iii) they can selectively alter the phenotype of cells in which they are expressed. In the case of the β_2 integrin-binding intramers, it is likely that they do this by localizing and binding their target at the plasma membrane.

Future Prospects

Intracellular aptamers provide diverse and versatile instruments that allow the targeted manipulation of cellular physiology. They can be used to control the intracellular function of proteins either by blocking ribosomal translation or by modulating the gene product directly. The strategy of using intramers as specific inhibitors of endogenous proteins has significant advantages over the most frequently applied current approaches in functional genomics. Because nucleic acids are naturally located inside cells, intramers are pre-adapted to function in the intracellular context. Furthermore, intramers can be applied in diverse cellular compartments, including the cytoplasm, where most antibodies or scFv would lose their function, presumably due to incorrect folding in the reductive intracellular environment. As the in vitro selection approach is highly robust and requires no intermediate amplification steps in living cells, it has been possible to adapt the process to fully automated selection protocols which might be further optimized to screen for hundreds of aptamer effectors in a few days.[69] In principle, the powerful methods which are established for the high-throughput decryption of the genetic information could now be used to analyze the encoded functionalities in a similarly efficient manner. Intramers selected either in vitro or in vivo could offer a convenient and time-saving method to quickly gain insights into the biological role of the numerous, so far poorly characterized intracellular proteins.

We dedicate this paper to Professor Ernst-L. Winnacker. The research described herein was supported by the Deutsche Forschungsgemeinschaft, the European Union, the Fonds der Chemischen Industrie, Boehringer Mannheim, and the Consortium der Elektrochemischen Industrie, München. M.F. extends his most sincere thanks to all of the co-workers and collaborators who have contributed to this project over the past 7 years. Their names appear throughout the references.

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